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Effect of dietary lysine and genetics on indices of energy and protein metabolism in rainbow trout and alterations in the mitochondrial proteome in broilers fed a lysine-deficient diet

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Thesis submitted to the
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at West Virginia University
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for the degree of

Master of Science
in
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Abstract

Effect of dietary lysine and genetics on indices of energy and protein metabolism in rainbow trout and alterations in the mitochondrial proteome in broilers fed a lysine-deficient diet.

Stephanie K. Pomeroy

How an organism reacts to consuming a lysine deficient diet was examined in two different circumstances. First, the responses of ten different genetic lines of rainbow trout were observed while consuming a lysine-deficient (LD) diet, used to mimic a soybean meal-containing diet. The aquaculture industry is investigating the incorporation of soybean meal into carnivorous fish diets to alleviate the need for fishmeal. If soybean meal is incorporated into the diet, an assessment of the genetic strains of fish used in the aquaculture industry today must be performed since differences in genetics have been shown to effect growth. When compared to rainbow trout consuming a lysine-adequate (LA) diet, those consuming the deficient diet had a significant reduction in weight gain ($P<0.01$), average daily feed intake ($P<0.01$) and feed efficiency ($P<0.0001$), while having increased hepatosomatic index ($P<0.01$) and lipid content ($P<0.01$). The fish consuming the LD diet had no differences in indicators of lysine metabolism ($P>0.05$) when compared to those consuming the LA diet. These data indicate that a LD diet was successfully constructed. When the ten families consuming the LD diet were examined, there was variation in final weight ($P<0.0001$), feed intake ($P<0.0001$) and feed efficiency ($P<0.0001$). There was also trend for variation in oxygen consumption ($P=0.068$) among families, and there was no difference in indices of lysine metabolism among families. These data indicate that there is some genetic component influencing the ability of the fish to adapt to a LD diet, in that some families of fish out-perform others when consuming this diet. Lysine is degraded in mitochondrial matrix, but the mechanism of transport into the matrix is unknown. Second, changes in the mitochondrial proteome of broilers adapted to a LD diet were assessed. Two transport proteins were affected by the LD diet. Broilers consuming the deficient diet exhibited a 50% reduction in the expression of a voltage-gated anion-selective channel and a voltage-gated potassium channel compared to those consuming a LA diet, although it is unlikely that lysine is transported via either of these proteins. The consumption of the LD diet also impacted many aspects of metabolism. Glutamine synthase expression was decreased over 95% in the chickens consuming the LD diet, compared to the LA diet. Electron transport chain (ETC) components were also altered. Broilers consuming the LD diet had over a 30% reduction in NADH dehydrogenase expression, or Complex I, and almost a 90% decrease in the expression of ATP synthase, or Complex V of the ETC. The reduction in proteins involved in the ETC may contribute the reduction in growth ($P<0.05$) seen in the broilers consuming the LD diet. Proteins involved in fatty acid oxidation were also affected by the LD diet, as Acyl CoA dehydrogenase was reduced about 75%. These data indicate that a LD diet affects many aspects of metabolism, possibly explaining the reduction of growth typically seen with a lysine deficiency.

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Table of Contents

List of Equations, Tables and Figures

Chapter I

Chapter II

Chapter III

Chapter I. Literature Review

Animal Production

In animal production, a main focus of research has been to improve growth and feed efficiency, thus maximizing profit. Feed efficiency is defined as weight gained per feed consumed, and is one of the most economically important production traits (Lin, 1980). Enhancing feed efficiency, or how an animal is capable of incorporating feed into body weight, will decrease production costs. Feed costs represent the greatest variable cost (~70%). In the poultry industry, most animals are fed *ad libitum* which may lead to over-consumption of feed, resulting in unfavorable energy situations. Specifically, energy will be directed away from growth and towards the degradation of excess nutrients, resulting in decreased feed efficiency, and increased feed costs. Optimizing feed efficiency will alleviate the impact of these costs by increasing mass gained per feed consumed. To impact feed efficiency, one must understand the factors that affect efficiency. Two primary factors affecting feed efficiency are the animal's nutrition and its genetics.

Lysine Nutrition

One method of optimizing feed efficiency is to maintain the proper proportion of nutrients in the diet, increasing the potential for protein synthesis. Protein synthesis is made possible by the twenty amino acids, which are protein building blocks. Lysine is an essential amino acid to a variety of species including chickens, swine, fish and humans. An essential amino acid is one which cannot be synthesized in the body, and must be obtained through the diet.

A corn/soybean meal based diet may be limiting in lysine. A limiting amino acid is one which is in shortest supply relative to its requirement. Consuming a diet where a limiting amino acid is below its requirement will ultimately restrict the animals' ability to synthesize body proteins. In typical production diets, lysine is first limiting for swine, and second limiting for chickens and fish. A lysine deficiency may develop without further supplementation to a corn/soybean meal based diet. Chickens (Latshaw, 1993) and fish (Walton et al., 1984) fed lysine-deficient diets had a decrease in weight gain and feed efficiency relative to those fed a lysine-adequate diet. So, as a result of this decreased growth, many production feeds are supplemented with lysine, either via feed grade lysine or soybean meal, to meet the requirement for maintenance and to optimize growth.

Lysine supplementation is a common practice in animal production industries where corn and soybean meal are the protein source. For poultry, lysine supplementation to meet the requirement leads to increased rate of growth, as well as muscle weight, while simultaneously improving feed efficiency (Tesseraud et al., 1999). Similar results were shown for swine. When lysine was supplemented to a corn-peanut oil diet to meet or exceed its requirement, rate of gain and muscle weight both increased, while also improving feed efficiency, when consuming a diet of normal protein levels (Vipperman et al., 1963). It is clear that lysine supplementation improves rate of gain and the efficiency of converting food energy into muscle protein in lysine limiting diets.

Ingested lysine is eventually integrated into the body's free amino acid pool. The free pool includes all free amino acids that are available for protein synthesis. Free lysine has two primary fates; either it can be degraded, and used as an energy source, or it can

be used for protein synthesis. As lysine is degraded, the free lysine pool is depleted, and less is available for incorporation into protein. The free lysine pool can only be restored by the ingestion of more lysine, or by the degradation of existing proteins. The turnover of body proteins contributes about 70-80% of the free amino acids to the total pool (Millward et al., 1975). Increasing the free lysine available for protein synthesis, such as by supplementation, may lead to increased growth and efficiency. In addition to lysine supplementation, the lysine pool may also be increased by decreasing the amino acids' rate of degradation. If the rate of lysine degradation is decreased, more will be available for protein synthesis. Decreasing lysine degradation under situations where lysine is first-limiting allows lysine to be conserved for protein synthesis.

Lysine conservation has been demonstrated previously. It was hypothesized that when an essential amino acid was completely lacking in the diet, the animal would act as if it were fed a protein-free diet (Said and Hegsted, 1970). This hypothesis proved true for most amino acids, however, rats fed a lysine-free diet lost considerably less body weight than those fed diets devoid of other essential amino acids. This result led to the concept that lysine is conserved, allowing adaptation to lysine-limiting diets. Chicks fed a diet devoid of lysine survived twice as long as those fed diets devoid of other essential amino acids (Ousterhout, 1960). Apparently, animals have adaptive mechanisms which conserve some amino acids when they are in short supply, and these adaptive mechanisms appear to be most pronounced for lysine (Chu and Hegsted, 1976). The potential conservation of lysine may lead to the optimization of its utilization for protein synthesis.

Lysine Metabolism

Lysine catabolism by the saccharopine-dependent pathway is thought to be the predominant pathway for lysine degradation in mammals (Broquist H.P., 1991). In this pathway, lysine is converted to saccharopine in a NADPH-dependent step by the enzyme lysine α-ketoglutarate reductase (LKR) (E.C. 1.5.1.8). This step involves the condensation of lysine with α-ketoglutarate (α-KG) to form saccharopine (Figure 1). Next, the saccharopine is oxidized by a dehydrogenase to produce α-aminoadipate-γ-semialdehyde and glutamate in a NAD^+-dependent reaction. This reaction is catalyzed by the enzyme saccharopine dehydrogenase (SDH) (E.C. 1.5.1.9; Figure 1). It was later discovered that LKR and SDH actually occur on a single protein, a bifunctional aminoadipic semialdehyde synthase, or AASS (Markovitz et al., 1984).

Although there is intriguing evidence supporting alternative routes of lysine degradation, it is well accepted that the LKR-dependent pathway is the primary route of degradation. Consistent with the LKR-dependent pathway serving as the primary route of lysine degradation is the human disorder hyperlysinemia. Hyperlysinemics display a decreased ability to degrade lysine and usually a diminished LKR activity, resulting in mental retardation and elevated lysine in the blood and urine (Dancis et al., 1969).

The LKR pathway of lysine degradation has been observed for a number of species. It is the primary method of degradation in vertebrates. LKR activity has been demonstrated in mammals such as, the rat (Higashino et al., 1965), mouse (Papes et al., 1999) and chicken (Wang et al., 1973). The LKR pathway is assumed to be the predominant pathway of lysine degradation in most mammalian species. The

characterization of LKR activity in developing maize endosperm provided evidence that this pathway was also functional in plants (Arruda et al., 1982).

In plants, evidence indicates that LKR is regulated by post-translational modification (Stepansky et al., 2006). In the developing tobacco seed, lysine regulates its own concentration by stimulating LKR activity. This stimulation occurs by a specific signaling cascade mediated by calcium and protein phosphorylation (Karchi et al., 1995). Some evidence for post-translational control in mammals has also been reported. Mice fed a high protein diet had an increased AASS mRNA ratio, but AASS protein abundance was unchanged (Kiess, 2006). Also, in chickens fed a lysine-deficient diet, there was an increase in LKR mRNA, but this also did not translate to greater protein abundance. These data are consistent with post-translational modification, potentially via phosphorylation, as the primary means of regulating LKR activity (Kiess, 2006).

Lysine α-ketoglutarate reductase was first discovered in rat liver (Higashino et al., 1965). It was later determined that within the liver, LKR was specifically located in mitochondria (Noda et al., 1978). Next, LKR activity was further examined in mitochondrial sub-fractions and it was discovered that LKR was located only in the mitochondrial matrix (Blemings et al., 1994). The fact that the enzymes responsible for lysine degradation are located specifically in the mitochondrial matrix indicates that lysine must first be transported into the mitochondrion, through its inner membrane, in order for its catabolism to take place.

It was hypothesized that the rate of lysine transport into the matrix limits its metabolism. Yet, there has been very little research into the mechanism of lysine transport into mitochondrion. There has been some speculation that the mechanism of

transport for lysine is similar to that of ornithine. Both lysine and ornithine are cationic amino acids. Ornithine is a non-essential amino acid structurally similar to lysine, only differing in one methylene group in the side-chain. The two amino acids also share other commonalities. Ornithine transcarbamylase and ornithine aminotransferase, the enzymes responsible for ornithine "degradation", are also found exclusively in the mitochondrial matrix. Also, since a transport limitation to ornithine catabolism has been shown (Hommes et al., 1982), there has been speculation of similar limitations for lysine (Blemings et al, 1994).

There has also been evidence that the two amino acids are transported via similar mechanisms. Ornithine is transported into the matrix via a hydrogen antiporter. When both lysine and ornithine mitochondrial uptake activities were measured simultaneously, they mutually inhibited each other, leading to the assumption that transporters for lysine uptake are the same as ornithine in rat liver mitochondria (Hommes et al., 1982).

Other possible alternatives to lysine transport into mitochondria have been investigated. Two mitochondrial basic amino acid transporters in *Arabidopsis thaliana* have been examined to better understand how the amino acid arginine, also a basic amino acid, is transported into the mitochondrion for degradation (Hoyos et al., 2003). One of the transporters was purified and determined to have a molecular mass of about 37 kDa. The identification of this transporter may give some insight into the mechanism of lysine transport.

With respect to lysine metabolism, two questions that clearly remain are 1) "is LKR phosphorylated?" and 2) "how is lysine transported into the matrix?". Answers to these questions will prove useful in understanding the regulation of lysine catabolism.

Beyond an understanding of lysine metabolism, to increase the efficiency of lysine use for protein synthesis, an appreciation of the role of genetics and genetic variation would be helpful. A genetic component to the lysine requirement has been demonstrated, since some genotypes are less sensitive to a lysine deficiency compared to others (Tesseraud et al., 1999).

Genetics and Growth

In many production species, growth has been one of the targets for genetic improvement (Hershberger et al., 1990). Recent research has been focusing on improving genetic lines to increase positive attributes. Many species have been investigated thus far for the degree of genetic control, or heritability, of these traits. In pigs, distinct genotypes exhibited differences in growth rate, feed efficiency and utilization of amino acids (Fabian et al., 2003). Similar results were shown for broiler chicks, where different genetic lines showed variation in growth performance (Tesseraud et al., 1999). There is also research investigating the influence of genetics on improved growth in aquacultured species, such as Salmonids.

In aquacultured species, superior growth has been used as a selection criterion with the assumption that it will lead to an improved feed efficiency (Thodesen et al., 2001). A high positive genetic correlation has been shown between average daily gain and average daily feed intake in European whitefish. Simultaneously selecting for increased daily gain and decreased feed intake achieved the greatest genetic gains (Quinton et al., 2007). There is also a variation among genetic lines of Atlantic salmon

relative to average daily feed intake, growth rate and feed efficiency (Thodesen et al., 2001).

From the viewpoint of genetics, it is noteworthy that commercially available strains of rainbow trout used by the nation's producers were selected for growth using fishmeal based diets. Plant proteins have recently been included in the diets of carnivorous fish to decrease the amount of fishmeal incorporated into the diet. This inclusion of a plant protein may alter the efficiency of the strains available. When soybean meal replaced more than 50% of the fishmeal within the diet, detrimental effects on performance were observed (Chou et al., 2004, Elengovan and Shim, 2000). Since the inclusion of plant protein can negatively affect feed efficiency, strains selected for superior growth may also change. Therefore, the effect of genetics on feed efficiency needs to be re-evaluated for rainbow trout while consuming a plant-protein containing feed.

Lysine and Aquaculture

The aquaculture industry is expanding. From 1987 to 1997, global production of farmed fish more than doubled in weight and value, accounting for one fourth of all fish directly consumed by humans (Naylor et al., 2000). Although this is great for the industry, it's been speculated that the future supply of fishmeal, the primary protein source for carnivorous fish, will not meet its demand. The annual global fishmeal production has been fairly consistent for the past 15 years and is not expected to increase (Cheng et al., 2003). A considerable amount of protein is used to construct diets for

aquaculture feed because the protein requirement for fish is two to four times that for other vertebrates (Wilson, 2002).

A common way to alleviate the need for fishmeal is by the incorporation of plant-based protein into the diet. Plant products, such as soybeans, have been investigated as a replacement for fishmeal since the 1970s (Quinton et al., 2007). Soybean meal has been a top replacement choice because of its high content of available protein, reasonable price and steady supply. However, soybean meal contains up to 30% indigestible carbohydrates, which may damage the digestive tract of carnivorous fish, the severity of which may depend on the fish species (Refstie et al., 2000).

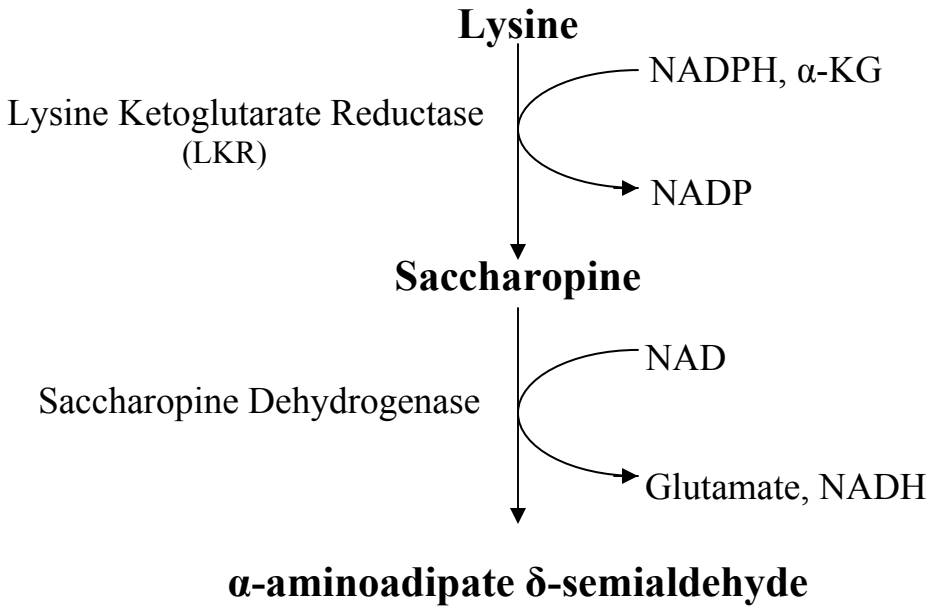
Numerous studies have investigated the response of aquacultured species to a diet containing soybean-meal. Rainbow trout consuming a diet that had 60% of the protein coming from soybean meal exhibited a significant decrease in growth after 28 days compared to a fishmeal control diet (Refstie et al., 1997). Atlantic salmon exhibited a decrease in weight gain, but not rainbow trout, when consuming a diet with 30% of protein coming from soybean meal, compared to those consuming a fishmeal-based diet (Refstie et al., 2000). These results indicate that there is a species specific response while consuming a soybean meal-containing diet; with rainbow trout out-performing Atlantic salmon.

Beyond the indigestible carbohydrate, another potential explanation for the decreased growth correlated to the incorporation of soybean meal into the diet is that, relative to fishmeal, many plant proteins have a reduced lysine content (Cheng et al., 2003). As stated previously, a lysine deficiency leads to decreased growth, so it may be necessary to supplement these diets with amino acids, such as lysine, to ensure adequate

nutrition. Fish fed a plant-protein based diet gained less weight than those consuming a fishmeal-based diet, yet when lysine was supplemented at 0.4% or higher, the weight gain was not significantly different between diets (Cheng et al., 2003).

A possible drawback to the inclusion of soybean meal into the diets of carnivorous fish is that the genetic strains that are commercially available were chosen based on their performance while consuming a fishmeal-based diet. To correct for the change in protein source, selection programs must account for the change in the genotype by environment interactions (Quinton et al., 2007). The significant effect of family on indices of performance, such as feed efficiency, may not be the same under different nutritional circumstances. From an industry standpoint, it would be beneficial to estimate genetic potential while consuming a soybean meal containing diet.

Figure 1. Initial Enzymatic Reactions of LKR pathway



Statement of the Problem

A main focus of research in animal production is to improve growth and feed efficiency. Amino acids are essential for growth because they are used to synthesize body proteins. Lysine is an essential amino acid which is limiting in many production feeds. Consuming a diet limiting in lysine would limit growth. Due to the importance of dietary lysine, improving the efficiency of lysine use for protein synthesis would be beneficial.

Improving lysine efficiency can also be achieved by genetic selection. Efficiency has been one of the targets for genetic selections in the aquaculture industry. The expansion of the industry is leading to the incorporation of more plant-based proteins into the diet of carnivorous fish, replacing a portion of fishmeal. Increasing plant proteins, such as soybean meal, in the diet has been shown to have negative effects on growth. One potential explanation is that, in relation to fishmeal, many plant proteins have reduced lysine content. Selecting fish with a reduced lysine requirement may ease the cost of supplementing lysine to plant-based feeds.

Understanding lysine metabolism is crucial to improving lysine efficiency. Decreasing the rate of lysine degradation should leave more free lysine available for protein synthesis. Lysine is primarily degraded by the saccharopine-dependent pathway. The first enzyme involved in lysine degradation is lysine α-ketoglutarate reductase (LKR). LKR has been localized specifically in the mitochondrial matrix of hepatocytes. It is the transport of lysine through the inner mitochondrial membrane which appears to limit its catabolism. Obtaining more information on lysine uptake by mitochondria may provide more insight into the regulation of lysine metabolism.

Chapter II

Effect of dietary lysine and genetics on indices of energy and protein metabolism in rainbow trout

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Keywords: Lysine, Rainbow trout, Genetics

Abstract

The objective of this study was to investigate the response of different genetic lines of fish to a lysine-deficient (LD) diet. Five families of rainbow trout previously evaluated for high feed efficiency and 5 selected for low feed efficiency were used. These 10 families were fed a LD, soy protein concentrate containing-diet. The highest and lowest feed efficiency families also received a lysine-adequate (LA) diet as a positive control. For each diet x family treatment combination, 5 replicates were used. As expected, feed efficiency and thermal growth coefficient (TGC) of fish fed the LA diet was better ($P<0.05$) than fish fed the LD diet. Hepatosomatic index (HSI) and lipid content were higher for fish consuming the LD diet compared to those consuming the LA diet. There was no effect of diet on indices of lysine metabolism, such as, lysine α-ketoglutarate reductase (LKR) activity, LKR mRNA abundance or lysine oxidation (LOX). There was a family effect on feed efficiency ($P<0.0001$), TGC ($P<0.0001$), and condition factor ($P<0.05$). There was a trend for a family effect on oxygen consumption ($P<0.07$) but no effect on ammonia excretion, HSI, lipid content, efficiency of nitrogen retention or indices of lysine catabolism. A single family had a numerically more favorable feed efficiency and thermal growth coefficient when fed the LD diet, indicating that it may possess an enhanced genetic potential for a reduced lysine requirement.

1. Introduction

Due to the recent expansion of the aquaculture industry, it has been speculated that the future supply of fishmeal, the primary protein source for carnivorous fish, will not meet its demand. To alleviate the need for fishmeal, plant protein sources, such as soybean meal, have been explored as a potential replacement since the 1970s (Quinton et al., 2007). Although soybean meal incorporation in the diet may lessen the dependence on fishmeal, numerous studies have found a decrease in growth of carnivorous fish consuming diets containing soybean meal (Refstie et al., 1997, 2000).

One potential explanation for the decreased growth is that the incorporation of soybean meal into the diet of carnivorous fish increases the carbohydrate content of the diet, while decreasing the protein content. Also, relative to fishmeal, many plant proteins have a reduced lysine content (Cheng et al., 2003). Lysine is an essential amino acid in fish species, meaning they cannot synthesize it. If lysine is limiting in the diet, it will limit protein synthesis, thus limiting growth. Lysine supplementation to plant-based diets has been shown to improve growth in rainbow trout (Cheng et al., 2003). Also, since the inclusion of proper levels of essential amino acids is important to maximizing growth, it will also impact profit.

Another approach to maximizing growth is to improve feed efficiency, which has been the target of selection (Hershberger et al., 1990). A genetic influence on growth performance has been found in broiler chicks (Hulan et al., 1980, Tesseraud et al., 1999), pigs (Fabian et al., 2003), and fish (Quinton et al., 2007). Different genotypes of broiler chicks respond differently while consuming diets with decreased protein levels (Smith et al., 1998). There is also evidence that there is a genetic effect on performance related to

lysine metabolism. A genetic effect has been demonstrated for growth in broiler chicks consuming a diet that meets the lysine requirement (Holsheimer et al., 1992) or exceeds the requirement (Bilgili et al, 1992), yet no genetic effect on growth was detected in chicks consuming a diet slightly deficient in lysine (Acar et al., 1991).

Little is known about lysine metabolism in rainbow trout. Understanding lysine's degradation may prove important in understanding any genetic effects on lysine metabolism, as well as optimizing lysine utilization, potentially decreasing the need for lysine supplementation. Lysine catabolism by the saccharopine-dependent pathway is thought to be the predominant pathway for lysine degradation in mammals (Broquist, 1991). In this pathway, lysine is converted to saccharopine in a NADPH-dependent step by the enzyme lysine α-ketoglutarate reductase (LKR) (E.C. 1.5.1.8). This step involves the condensation of lysine with α-ketoglutarate (α-KG) to form saccharopine. Next, the saccharopine is oxidized by a dehydrogenase to produce α-aminoadipate-γ-semialdehyde and glutamate in a NAD^+-dependent reaction. This reaction is catalyzed by the enzyme saccharopine dehydrogenase (SDH) (E.C. 1.5.1.9).

Genetic variation for lysine degradation in rainbow trout is not well studied. Recently, a genetic effect on LKR mRNA abundance, but not on LKR activity or lysine oxidation has been reported (Higgins et al., 2005). This is an important variable to investigate, especially in fish consuming a plant-protein based diet, since it is potentially deficient in lysine. There has been evidence that there is a genetic influence on the ability of a broiler chick to use lysine for protein deposition in limiting situations (Tesseraud et al., 2001). It would be beneficial to examine if this is also true in rainbow trout.

From the viewpoint of genetics, it is noteworthy that commercially available strains of fish used by the nation's producers were selected for optimal growth using fishmeal based diets. Since the replacement of fishmeal by soybean meal is desirable, the performance of current strains should be re-evaluated while consuming a plant-based feed. Therefore, the objective of this study was to evaluate performance and lysine metabolism of several strains of rainbow trout fed a lysine-deficient diet.

2. Materials and Methods

2.1 Family selection

The National Center for Cool and Cold Water Aquaculture (NCCCWA) generated background information on 105 different full-sibling families of rainbow trout (*Oncorhynchus mykiss)* fingerlings. The fingerlings were grown from approximately 2 to 10 grams while consuming a commercial fishmeal-containing diet. Feed intake and body weights were then collected over a period of 2 months for each family and were used to determine feed efficiency. Using this information the 5 most and least efficient families were selected for further study (Table 1).

2.2 Experimental Design

Fish (approximately 100 grams) were randomly allotted to 30 gallon tanks at the NCCCWA with 6 fish per tank. Each of the 10 families had 5 replicates, giving a total of 50 tanks. These tanks were fed a plant-protein based diet first limiting in lysine to mimic the situation in a diet containing soybean meal. The diet was constructed at the Hagerman Fish Experiment Station to contain 1.19% dietary lysine (Table 2). The most and least efficient families were also fed a plant-protein based lysine adequate diet (2.9%, Table 2), with 5 replications per family. Water was kept at 14^{o}C, and a recirculating system replaced 8 liters of water per minute. Also, dissolved oxygen was maintained around 12 mg/L. All tanks were exposed to as 12:12 light:dark cycle/

At the start of the experiment, 2 fish from each of the 60 tanks were marked with a fluorescent colored elastomer eye tag (NMT, Shaw Island WA) for individual identification. Fish were hand fed their respective diets to apparent satiation at 0700 and

1400 hours daily for 6 days per week. All tanks were given a 7 day acclimation period, after which initial weights and lengths were recorded. One fish from each tank (5/family) was euthanized to enable the comparative slaughter technique for changes in composition. After euthanasia, the gastrointestinal contents of the GI tract of each fish were removed. The fish were then placed on ice and transported back to West Virginia University, where they were stored at -20^{o}C until analyzed for proximate analysis. The remaining fish were grown to a final weight of about 200g (227 $\pm$ 94 g) over a period of approximately 3 months. All fish were fasted 24 hours prior to harvest. Body weights and lengths were recorded for all 300 fish. The gastrointestinal contents of the two fish per tank with the elastomer tags were flushed with phosphate buffered saline. These fish were then placed on ice and transported back to West Virginia University, where they were stored at -20^{o}C until analyzed. Livers of the remaining fish were weighed. From two fish per tank, a portion of one liver was snap frozen in liquid nitrogen, and then stored at -80^{o}C, until RNA extraction. The second liver was cut in half, and each portion was placed in ice cold H buffer (5 mM Hepes, 5 mM 2-Mercaptoethanol, 1 mM EGTA, 220 mM Mannitol, 70 mM Sucrose, 0.05% (w/v) bovine serum albumin, pH 7.4) for LKR activity, and lysine oxidation analyses. Both assays were performed on fresh liver samples on the day of euthanasia. Blood samples were taken from two fish per tank for the analysis of non-esterified fatty acids (NEFA), which was also performed on harvest date.

2.3 Performance Analysis

All tanks were hand-fed to apparent satiation twice daily, and feed intakes were calculated weekly. At study termination, all weekly intakes were totaled to determine total tank intake. Body weights and lengths at the start and end of the study were taken for all fish to obtain measurements of growth and weight gain per tank. Individual weight gain was calculated from the initial and final weights obtained from the two fish per tank which were elastomer tagged. Feed efficiency and thermal growth coefficient (TGC; equation 1), which was first proposed by Iwama and Trautz (1981), were calculated. Hepatosomatic index (HSI) was calculated as liver weight divided by the total body weight. Oxygen consumption was calculated based on the equation utilized by Zakes et. al. (2003; equation 2). Briefly, three days prior to harvest, a Plexiglas disk was placed onto each tank, covering the water surface, to eliminate the oxygen transfer between the water and air. The following day, dissolved oxygen was measured with a handheld oxygen meter. The dissolved oxygen concentration was measured in a tank which did not contain any fish, while dissolved oxygen concentration in the effluent of the tank was measured for each individual tank. The oxygen probe was inserted ¾ of the length of the standpipe, which is assumed to have the same water quality as the rearing unit (Colt, 1991). Dissolved oxygen concentrations were taken hourly from 0730 to 1330 to ensure an elevation in oxygen consumption after eating, since there is an increase in oxygen consumption 2-3 hours after the start of feeding, followed by a stabilization about 6-7 hours after feeding (Zakes, et. al., 2003). Water flow was determined by collecting the inflow of each tank for 15 seconds to determine rate. Also 2 days prior to harvest, ammonia excretion was determined by collecting 50 mL of water from the outflow of the

standpipe from each tank hourly from 1130 to 1530 in a conical vial. Water samples were filtered through a 25 mm GD/X syringe filter device with a pore size of 1.2 µm (Whatman, Kent Me) and analyzed on Dionex ICS-90 Ion Chromatography System (Dionex, Sunnyvale CA). For proximate analysis, the fish with the elastomer tags were frozen in liquid nitrogen and then homogenized in a blender to a powder. The ground fish samples were used to determine protein and lipid content, as well as amino acid composition. Each sample was analyzed for protein and lipid in duplicate. Crude protein was determined by Kjeldahl analysis (AOAC, 2006) on a Kjeltec Auto 1030 Analyzer (Foss North America Inc., MN) and lipid content was determined by ether extraction using the Soxhlet method. Based on the proximate composition of the fish sampled at the beginning and end of the study, as well as proximate analysis of test feeds, efficiency of nitrogen retention was calculated.

2.4 Lysine α-Ketoglutarate Reductase Assay

Hepatic LKR activity was determined on fresh trout liver using the method of Higgins et al. (2005). Liver was homogenized using a Potter-Elvejhem device in enough H buffer to make a 25% (w/v) solution. Lysine α-ketoglutarate reductase activity was measured by the lysine-dependent NADPH oxidation at 340 nm. Enzyme activity was measured by the addition of 25 µL of homogenate to a cuvette containing 875 µL of buffer (127.5 mM Hepes, 114.75 mM mannitol, 38.25 mM sucrose, 4.25 mM 2-mercaptoethanol, 0.0425% bovine serum albumin, 0.21 mM NADPH, 12.75 mM α-ketoglutarate, and 0.05% (v/v) Triton-X 100) for blank measurements. A second buffer was constructed with the exact contents of the blank buffer, with the addition of 50 mM

L-lysine. Each sample was run in the presence and absence of lysine. The reported Michaelis Constant (K_m) of rainbow trout LKR was 7.3 mM for lysine and 0.5 mM for α-ketoglutarate (Walton et al., 1984). Thus, the concentrations of lysine and α-ketoglutarate will provide conditions near V_{max}. Tubes were covered with parafilm, and inverted to mix. Absorbance was measured in duplicate using a Beckman Coulter DU 640 spectrophotometer.

2.5 Lysine Oxidation Assay

Hepatic lysine oxidation was measured by determining the recovery of $^{14}CO_2$ from [U-^{14}C] L-lysine. This procedure was described in rats (Blemings et al., 1998) and then modified for rainbow trout (Higgins et al., 2005). A base trap was constructed by the addition of 1 part ethanolamine to 2 parts methyl cellosolve. A total of 0.5 mL of base trap was added to an Eppendorf tube, which was suspended in a 25 mL Erlyenmyer flask. A 25% (w/v) liver homogenate was made with fresh liver and H buffer. For each reaction, 1 mL of homogenate was added to a 25-mL Erlyenmyer flask, which contained 1 mL of lysine oxidation buffer (LOB) (10 mM L-lysine HCl, 10 mM Hepes, 3mM $MgCl_2$, 0.2 mM EDTA, 182 mM mannitol and 61 mM sucrose, final concentrations) which had previously been tempered to 25^{o}C in a water bath for 5 minutes. The solution was then incubated in the 25^{o}C water bath for 30 minutes, while oscillating at 100 osc/min. To terminate the reactions, 0.5 mL of 35% perchloric acid was injected via an 18 gauge needle through the serum cap covering the flask. Flask then remained in the water bath for at least 180 additional minutes, to ensure maximal recovery of the $^{14}CO_2$. After incubation, the Eppendorf tube was placed in a plastic scintillation vial along with

17 mL of Bio-Safe II liquid scintillation fluid. After the solution was mixed via vortex, the radioactivity was measured using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter Inc, Somerset, NJ). Each liver was measured in duplicate.

2.6 Real-time PCR Analysis

Real time RT-PCR was used to estimate the abundance of LKR mRNA using acidic ribosomal protein (ARP) as a reference gene. The mRNA was measured as the bifunctional enzyme α-aminoadipate δ-semialdehyde synthase as previously described by Higgins et. al. (2005). The RNA was first isolated from the livers stored at -80 ^{o} C. Two hundred mg of liver was homogenized in 2 mL of Trizol reagent (Invitrogen); the homogenate was divided into two equal parts and incubated at room temperature for 10 minutes. Chloroform (200 μL) was added, and samples were incubated at room temperature for 15 minutes, and then centrifuged for 15 minutes at 14,000 x g at 4^{o}C. The RNA in the supernatant was back-extracted with acid phenol:chloroform (5:1), and then again centrifuged. The supernatant was collected, and back-extracted against acid:phenol:isoamyl alcohol (25:24:1) and centrifuged again. The resulting supernatant was collected, and RNA was precipitated with isopropanol, and pelleted by centrifugation. The RNA was washed twice with 1 mL of 70% ethanol, and air dried. The final pellet was resuspended in nuclease free water. The RNA was quantified and qualified using the A_{260}:A_{280} ratio, and diluted to a concentration of 1 μg/μL. The cDNA was prepared following DNase (Promega) treatment using random primers (Invitrogen) and M-MLV reverse transcriptase (Promega) according to manufacturer's instructions. Resultant cDNA was diluted 1:4 with nuclease free water. The diluted cDNA was added

to a 20 μL total reaction which included 10 μL 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μM forward ARP primer (5'-GAA AAT CAT CCA ATT GCT GGA-3') and 1.25 μM reverse ARP primer (5'-CTT CCC ACG CAA GGA CAG A -3') or 0.625 μM forward AASS primer (5'-GCG AGT GCT ACT ACT GGG TTC-3') and 0.625 μM reverse AASS primer (5'-CCT CTG CCT GGG TCA ACA AC-3'). The real time PCR procedure was performed using a BioRad ICycler IQ Detection System. The procedure began with a "hot start" at 95^{o}C for 5 minutes, followed by a cycle of a 95^{o}C denaturing step for 30 seconds, a 57^{o}C annealing step for 30 seconds, and followed by a 72^{o}C extension step for 30 seconds. This cycle was repeated a total for 40 times. A melt curve analysis was then performed to assess the amplification product.

The liver with the highest A_{260}:A_{280} ratio for each tank was chosen to determine LKR mRNA abundance. Each sample was run in triplicate for both the LKR and ARP primers on a 96-well plate. A pooled sample, which contained cDNA samples from all tanks, was included in triplicate for both primers for each plate analyzed. Primer efficiencies were determined from the slope of the regression line of the log of the cDNA concentrations versus the Ct value by the equation $E=10^{(-1/Slope)}$. Efficiency plates for the primer pairs were analyzed, and an acceptable efficiency for each was obtained, LKR with an efficiency of 1.99 and ARP with an efficiency of 1.96. The efficiencies were used to calculate the relative mRNA abundance using the "efficiency corrected relative expression" equation (Equation 3; Pfaffl, 2001).

2.7 Statistical Analysis

Statistical significance was assessed using the analysis of variance procedure of General Linear Models PC-SAS. A completely random design was used. The main effects of diet and family were tested. For the effect of diet, the experimental unit was the tank mean for each variable. When differences existed ($P<0.05$), means were separated by the least square means procedure. To ensure that the difference was due to diet, diet by family interactions was also investigated. To test for the effect of family, the experimental unit was also the tank mean for each variable. When significant differences occurred ($P<0.05$), means were separated by the least squares mean procedure, and then adjusted for multiple comparisons using the Tukey's studentized range test.

3. Results

3.1 Diet Effects

Considering only the two strains fed the lysine adequate and lysine deficient diets, no differences in initial weights were detected (Table 3). However, average daily feed intake was significantly increased in fish consuming the lysine adequate (LA) compared to the lysine deficient (LD) diet (Table 3). Also, average daily gain (ADG) of fish consuming the LA diet was 72% higher ($P<0.05$) than those consuming the LD diet (Table 3). Feed efficiency was higher ($P<0.05$) for fish fed the LA diet (Table 3). Thermal growth coefficient and condition factor also improved ($P<0.05$) for fish consuming the LA diet. Taken together, the preceding findings give confidence that the LD diet was in fact lysine deficient.

There was a significant effect of diet on hepatosomatic index (HSI), where the HSI was 49% greater for those fish consuming the LD diet (Table 4). Carcass composition differed, as there was a significant increase in lipid content in fish consuming the LD compared to the LA diet (Table 4). No differences in ammonia excretion or efficiency of nitrogen retention were detected (Table 4). No differences in indices of lysine metabolism (LKR activity, LKR mRNA abundance and LOX) were detected between the two diets (Table 4). There was also no difference in NEFA concentration or oxygen consumption between diets (Table 4).

3.2 Family Effect

There was a significant effect of family on numerous indices of performance. Final weight of fish was significantly affected by family, and average weights ranged

from 139 to 343 grams (Figure 2). Average daily feed intake (Figure 3), feed efficiency (Figure 4), thermal growth coefficient (Figure 5), and condition factor (Figure 6) were also affected by family. There was a trend for oxygen consumption to differ between families (P=0.068), although it was not significant (Figure 7).

Carcass composition did not differ among families, as there were no differences in HSI, efficiency of nitrogen retention or lipid content (Table 5). Ammonia excretion and NEFA concentrations also were not different (Table 5). There were also no differences in any indices of lysine metabolism, as LKR mRNA abundance, activity and LOX did not differ among families (Table 5).

It is interesting to note that the status of initial feed efficiency is not necessarily carried on through time (Table 6). If the final feed efficiency of each family was ranked from 1 to 10, with 1 being the numerically highest, or most favorable, 3 out of the 5 best final performers were initially chosen based on their poor efficiency status. Likewise, 3 of the 5 worst performers were chosen based on their initial superior efficiency, indicating that efficiency status did not remain constant with a change in protein source and/or change in stage of growth.

3.3 Correlation Analysis

Pooled sample data were analyzed for correlation analysis by the Pearson Correlation test. Average final weight was found to be positively correlated ($P<0.05$) with initial weight, average daily feed intake, average daily gain, feed efficiency, condition factor and lipid content (Appendix 1). Feed efficiency was also positively correlated ($P<0.05$) with efficiency of nitrogen retention, and negatively correlated

($P<0.05$) with HSI and lipid content (Appendix 1). Ammonia excretion was positively correlated ($P<0.05$) with HSI, while negatively correlated with NEFA (Appendix 1). Oxygen consumption was negatively correlated ($P<0.05$) with average final weight and positively with LOX, while there was also a negative correlation trend ($P<0.06$) with ammonia excretion (Appendix 1). Lysine α-ketoglutarate reductase mRNA abundance and LKR activity had no correlations with each other or any other variables (Appendix 1).

4. Discussion

This study indicates that a lysine-deficiency limits growth in rainbow trout (Table 3). Similar results have also been shown in poultry (Tesseraud et al., 1996, Latshaw, 1993), potentially due to a decreased rate of protein synthesis as a result of the lysine deficiency. The decrease in growth performance indicates that a lysine-deficient diet was successfully constructed, and genetic differences in response to this deficiency could be assessed.

The present data also demonstrate that fish consuming a lysine-deficient diet had an increased HSI compared to those consuming an adequate diet (Table 4). There have been numerous reports that whole liver weight remained constant between lysine-deficient broiler chicks and their adequate-fed counterparts (Carew et al., 2005, Tesseraud et al., 1996), as well as rats (Canfield et al., 1978). Although, when liver size was expressed as relative liver weights, such as HSI, it increased in rats consuming a lysine-deficient diet (Bahl and Venkitasubramanian, 1977). An increase in HSI may be explained by the occurrence of fatty livers seen with the consumption of lysine-deficient diets in rats (Aoyama et al., 1972, Singal et al., 1953, Viviani et al., 1964), where a lysine deficiency stimulated the accumulation of liver lipids. The accumulation of lipids is not a result of a single problem, but rather the sum of a number of effects (Viviani et al., 1966, Bahl and Venkitasubramanian, 1977). It is also possible that the increase in HSI is due to an increase in hepatic glycogen stores (Clark and Barron, 1972, Sidransky et al., 1969) caused by the use of amino acids in gluconeogenesis.

The increase in the HSI is correlated with the increase in whole body lipid composition (Table 4). Previous research has shown that consuming a lysine-deficient

diet increases body fat (Grisoni et al., 1991). One potential explanation for this increase in lipids is due to a potential deficiency of carnitine as a result of the lysine-deficiency. Carnitine is synthesized via transformation of metabolism of lysine and methionine (Tanphaichitr et al., 1976), and it is essential because it plays a role in intramitochondrial transport of fatty acids, as well as initiating β-oxidation of fatty acids (Borum and Broquist, 1977). Thus, a decrease in lysine could lead to a decrease in carnitine and an impaired ability to oxidize fatty acids.

Typically, an amino acid imbalance is thought to decrease the percentage retention of absorbed nitrogen (Bressani, 1962). Yet, present data indicates no change in efficiency of nitrogen retention with a lysine deficiency, nor do we detect a difference in ammonia excretion (Table 4). These results are in contrast to previous findings in which a decrease in efficiency of nitrogen retention and an increase in nitrogen excretion in one strain of pigs consuming a lysine-deficient diet (Rivera-Ferre et al., 2006). The present results may be a consequence of increased conservation or species differences or extent of imbalance. Although there was a decrease in nitrogen consumption, there was no change in excretion or efficiency of nitrogen retention with a lysine-deficiency. As opposed to excreting excess nitrogen, the fish consuming the lysine-deficient diet were potentially more efficient at incorporating nitrogen into protein accretion, rather than excreting it.

Next, observing changes in lysine metabolism under a lysine deficiency may help understand lysine metabolism. Other reports have indicated that nutritional status has affected lysine metabolism. Increased dietary protein has resulted in an increase in hepatic LKR activity (Blemings et al., 1998, and Hegsted, 1976). It has also been shown

that LKR activity decreased in rats and chickens fed a lysine-deficient diet (Kiess, 2006, Muramatsu et al., 1984). However, these data are in agreement with those experiments performed in rainbow trout, where there was no effect of dietary lysine on LKR activity (Walton et al., 1984) There was also no change in LOX, which supports previous findings (Higgins et al., 2005). It is interesting to note, that while neither LKR activity nor LOX changed with lysine deficiency, LKR activity was over 100-fold greater than the rate of lysine oxidation, which can also be used as a measurement of mitochondrial lysine uptake (Benevenga and Blemings, 2007). This data is consistent with the location of lysine catabolism, and that the transport of lysine into the matrix is the rate-limiting step.

In many production species, growth has been one of the targets for genetic improvement (Hershberger et al., 1990). In aquaculture, growth has been used as a selection criterion with the assumption that it will lead to an improved feed efficiency (Thodesen et al., 2001). It has been determined that there was a high positive genetic correlation between performance variables such as, average daily gain, average daily feed intake and feed efficiency in different aquacultured species (Quinton et al., 2007, Thodesen et al., 2001). Current data is in agreement with these results, showing a family effect on final weight, average daily feed intake, feed efficiency, thermal growth coefficient and condition factor, indicating there is a genetic factor linked to performance while consuming a LD diet. There is one family in particular; family 191, which had the highest numerical feed efficiency and thermal growth coefficient, indicating that it may be a superior genetic line while consuming a LD diet, and more efficient at conserving ingested lysine.

There was also a trend for a genetic effect on oxygen consumption ($P<0.07$), as well as a significant negative correlation between oxygen consumption and final weight ($P<0.05$). It has been demonstrated that this change is due to size; the smaller the animal, the greater its oxygen consumption per kg (Barlow, 1961). Higher oxygen consumption is indicative of the fish consuming the LD diet to be inefficient at utilizing food, explaining their decreased weight (Hoover-Plow and Nelson, 1985). Also, increased oxygen consumption has also been observed with increased activity (Spoor, 1946). So, it is also possible that the fish grew better because they were less active, thus more energy was available for growth.

Examining genetic effects on metabolic mechanisms may prove useful as selection criteria in strains of rainbow trout. Studying the genotypic effect on lysine metabolism could help in identifying strains of fish with increased performance when consuming a plant-based diet. Unfortunately, LKR has not been extensively explored in rainbow trout, and the effect of genotype on lysine degradation is unclear (Higgins et al., 2005). These data indicate that there was no genetic variation in lysine metabolism, since there was no family effect on LKR mRNA abundance, activity or lysine oxidation.

The current results indicate that different genotypes of rainbow trout respond differently to lysine deficiency, which may mimic the situation of consuming a diet including soybean meal. There is a family effect on final body weight and feed efficiency; two variables which are targets for optimizing profit. Utilizing a strain of rainbow trout known to exhibit superior growth while consuming a lysine-deficient diet, i.e. reduced requirement, could potentially reduce the cost of lysine supplementation to a soybean meal-containing diet. Therefore, genetic influences on growth and efficiency

should be taken into consideration when implementing nutritional programs with the incorporation of soybean meal as a protein source.

Equation 1. Thermal Growth Coefficient

$$TGC = [(W_f^{(1/3)} - W_i^{(1/3)})/(T \times t)] \times 100$$

Where W_f = final weight, W_i = initial weight, T = temperature in ^oC and t = time in days

Equation 2. Oxygen Consumption

$$\text{Oxygen consumption} = [(DO_{in} - DO_{out}) \times Q]/B$$

Where DO_{in} is dissolved oxygen flowing into the tank, DO_{out} is dissolved oxygen flowing out of the tank, Q is the water flow through tanks (L/h), and B is the fish biomass (kg)

Equation 3. Efficiency Corrected Relative Expression

$$\text{Ratio} = \frac{\frac{E_{LKR}^{Ct(\text{Pool LKR})}}{E_{LKR}^{Ct(\text{Sample LKR})}}}{\frac{E_{ARP}^{Ct(\text{Pool ARP})}}{E_{ARP}^{Ct(\text{Sample ARP})}}}$$

Where E = PCR Efficiency, C_t – Threshold Cycle, LKR is Lysine α-Ketoglutarate Reductase and ARP is Acidic Ribosomal Protein.

Table 1. Initial Efficiency Status

Family ID #	Initial Efficiency Status
14	Low
27	Low
68*	Low
113*	**High**
136	Low
137	Low
160	**High**
166	**High**
191	**High**
227	**High**

***Fed both the LD and LA diets**

Table 2. Diet Composition (g 100 g^{-1}) of experimental diets, calculated values for selected nutrients

Ingredients	Lysine Deficient	Lysine Adequate
Soy Protein Concentrate	11.13	10.52
Corn Gluten Meal	45.22	42.75
Wheat Gluten Meal	9.67	9.35
Wheat Flour	11.21	12.81
Fish Oil	18.29	18.29
Lysine-HCl	---	1.80
Di-calcium Phosphate	2.78	2.78
Choline Cl	0.60	0.60
Vitamin Premix 30	0.80	0.80
Trace Mineral Premix 3	0.10	0.10
Stay-C	0.20	0.20
Protein	45.2	45.0
Fat	20.0	20.0
Ca	.64	.64
P	.93	.93
Arg	1.81	1.74
His	1.15	1.10
Ile	2.09	2.00
Leu	6.02	5.72
Lys	**1.19**	**2.90**
Met	1.08	1.03
Cys	.78	.76
TSAA	1.86	1.79
Phe	2.72	2.60
Tyr	2.13	2.03
Thr	1.62	1.55
Trp	0.34	0.33
Val	2.40	2.29

Table 3. Effect of Diet on Indices of Performance of Rainbow Trout

Performance Variable	**LA Diet**	**LD Diet**	**SEM**
Average Initial Weight (g)	100.06	101.25	2.48
Average Final Weight (g)	273.75	199.93**	12.54
Average Daily Feed Intake (g)	1.81	1.41*	0.097
Average Daily Weight Gain ($g\ day^{-1}$)	1.92	1.11*	0.108
Feed Efficiency	1.09	0.79**	0.040
Thermal Growth Coefficient	1.39	0.88**	0.092
Condition Factor	1.40	1.13**	0.025

* $P < 0.01$, **$P < 0.0001$ compared to LA diet

Table 4. Effect of Diet on Carcass Composition and Lysine and Energy Metabolism

Variable	**LA Diet**	**LD Diet**	**SEM**
Hepatosomatic Index	1.00	1.49*	0.053
Lipid Content (%)	10.70	14.29*	0.812
Ammonia Excretion (mg Ammonia $kg^{-1}hr^{-1}$)	23.22	26.86	5.303
Efficiency of Nitrogen Retention (%)	31.88	31.00	3.47
LKR mRNA Abundance	1.14	0.74	0.172
LKR Activity (nmol NADPH consumed $kg^{-1}min^{-1}$)	3.68	3.72	0.356
LOX (pmol min^{-1} g $liver^{-1}$)	26.5	31.23	8.40
Oxygen Consumption (mg O_2 $kg^{-1}hr^{-1}$)	973.80	1073.78	126.86
NEFA Concentrations (mEq/L)	0.32	0.41	0.067

* Indicates a P value of <0.01

Table 5. Family Average of Variables (Non-Significant)

	Average	**SEM**
Hepatosomatic Index	1.52	0.33
Ammonia Excretion (g Ammonia $kg^{-1}hr^{-1}$)	21.82	2.51
Efficiency of Nitrogen Retention	22.05	13.39
Lipid Content (%)	14.54	3.19
LKR mRNA Abundance	1.10	1.70
LKR Activity (nmol NADPH consumed $kg^{-1}min^{-1}$	3.56	1.90
LOX (pmol min^{-1} g $liver^{-1}$)	45.29	59.68
NEFA Concentrations (mEq/L)	0.365	0.206

Table 6. Final Efficiency Status

Family ID #	Initial Efficiency Status	Final Efficiency Rank†
14	Low	**5**
27	Low	10
68	Low	**4**
113	**High**	**3**
136	Low	**2**
137	Low	6
160	**High**	7
166	**High**	9
191	**High**	**1**
227	**High**	8

†Final Efficiencies are ranked from 1-10, with 1 being the numerically highest, or highest efficiency.
*Bold entries represent the high performing families for initial or final efficiencies

Figure 2. Effect of Family on Final Weight

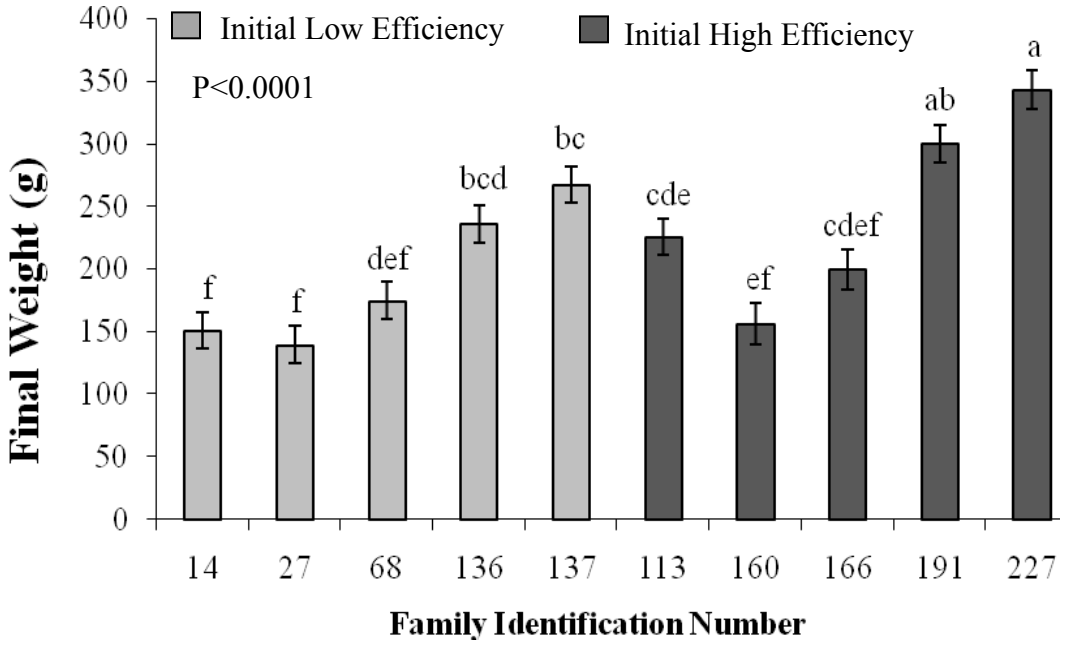


Figure 3. Effect of Family on Average Daily Feed Intake

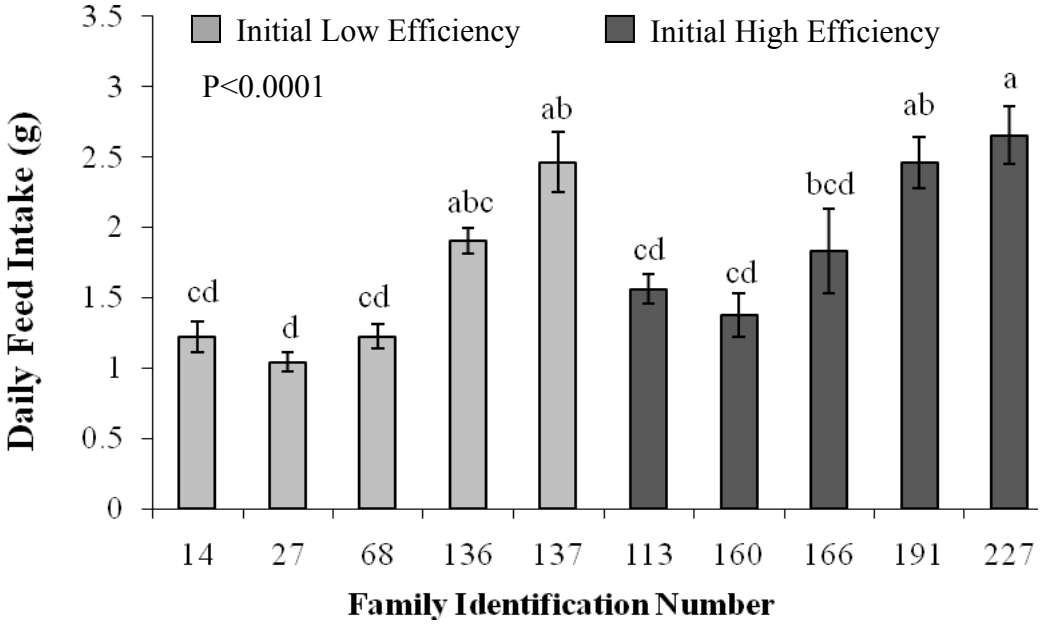


Figure 4. Effect of Family on Feed Efficiency

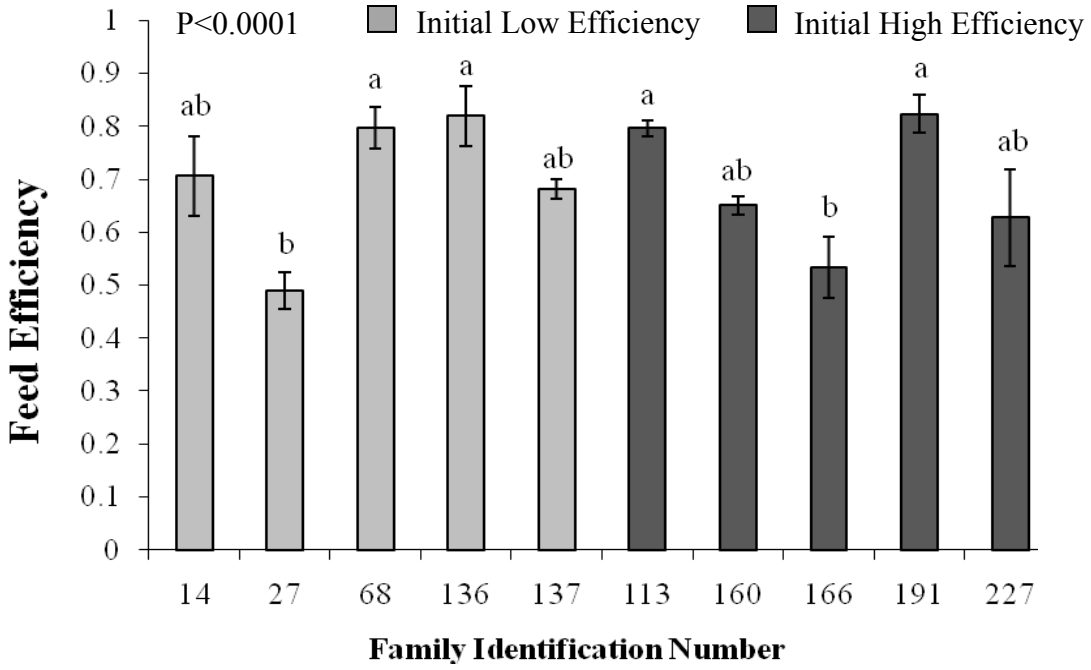


Figure 5. Effect of Family on Thermal Growth Coefficient

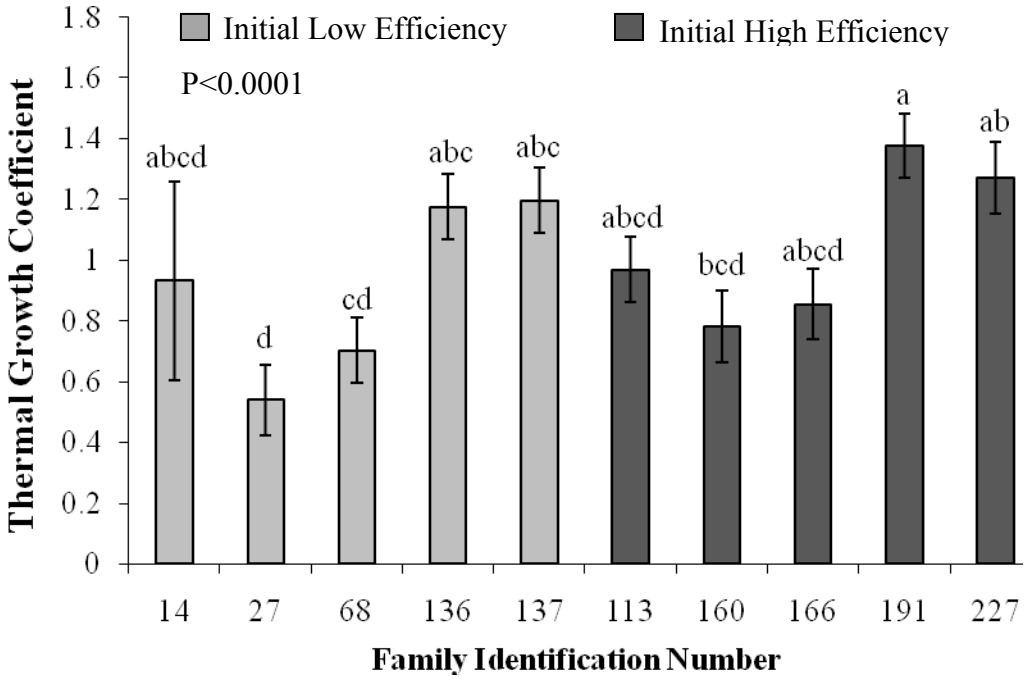


Figure 6. Effect of Family on Condition Factor

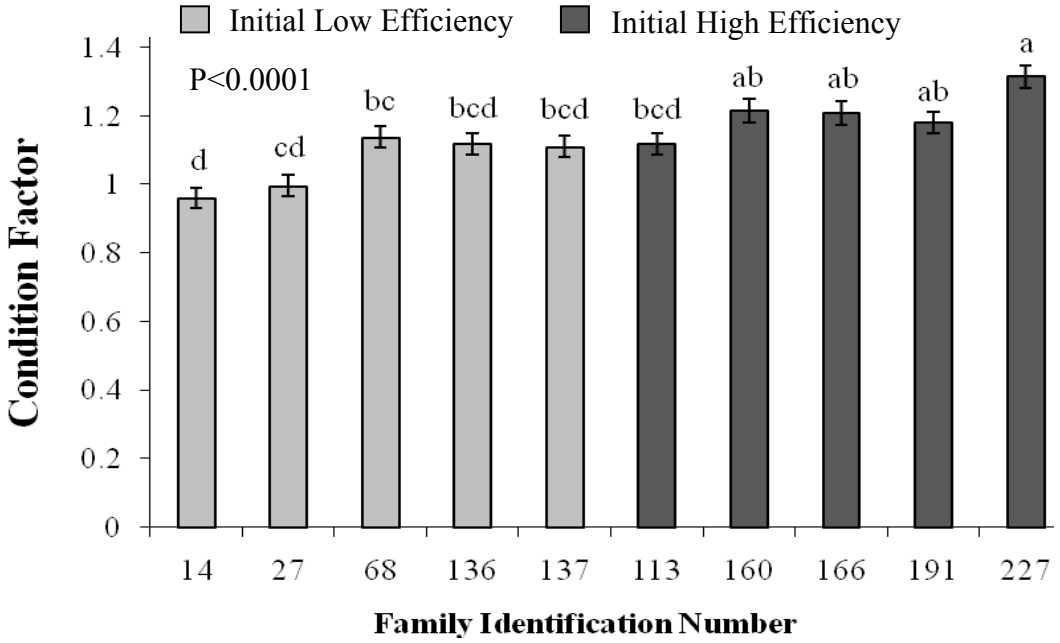
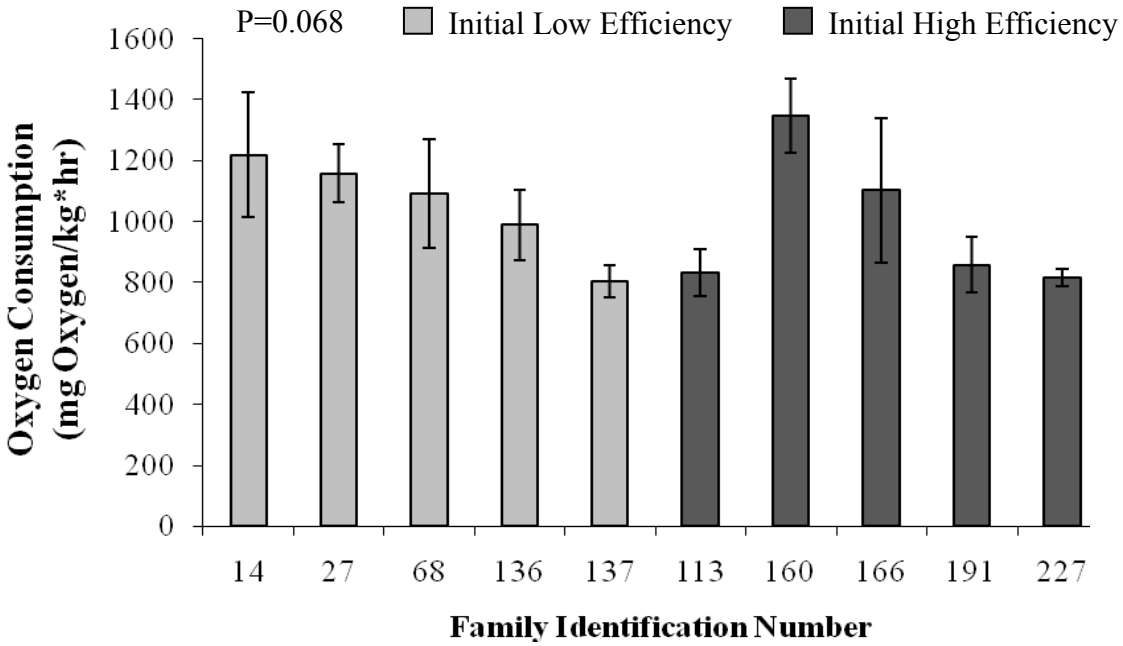


Figure 7. Effect of Family on Oxygen Consumption



Chapter III

Alterations in the Mitochondrial Proteome in Broilers fed a Lysine-Deficient Diet

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Abstract

In the liver, the site of lysine degradation has been shown to be the mitochondrial matrix. However, the mechanism as to how lysine is transported to this site is unclear. Examining the differences in the proteome of the inner membrane of the mitochondrion between broilers consuming a lysine-deficient (LD) and lysine-adequate (LA) diet may provide insight into a protein involved in lysine transport, as well as other protein changes affecting different aspects of metabolism. Two transport proteins in the inner mitochondrial membrane were affected by the LD diet. There was almost a 50% decrease in the expression of a voltage-gated anion-selective protein channel and about a 40% decrease in the expression of a voltage-gated potassium channel in the broilers consuming the LD diet when compared to the LA diet. However, it is unlikely that lysine is transported by either of these proteins. The consumption of the LD diet also impacted various aspects of metabolism. Expression of glutamine synthetase in broilers consuming the LD diet was less than 5% of its expression in broilers fed the LA diet. The electron transport chain (ETC) was also affected by the LD diet. There was a 30% decrease in NADH dehydrogenase, or Complex I, and almost a 90% decrease in ATP synthase, or Complex V, of the ETC in broilers consuming the LD diet compared to the LA diet. The reduction in proteins involved in the ETC, especially one involved in the synthesis of ATP, may aid in the explanation of the reduction of growth ($P<0.05$) seen with the lysine deficiency. Proteins involved in fatty acid oxidation were affected by the LD diet, as Acyl CoA dehydrogenase was reduced 75% compared to the broilers consuming the LA diet. These data indicate that a LD diet affects many aspects of metabolism, possibly explaining the reduction in weight gain typically seen with a lysine-deficiency.

INTRODUCTION

Lysine is an indispensible amino acid for non-ruminant animals (Latshaw, 1993). It is also the second limiting amino acid in poultry where soybean meal is the main protein source. Due to the limiting supply of lysine in the feed, lysine supplementation is a common practice in most poultry production industries. When lysine is supplemented to meet its requirement, chickens demonstrate increased growth and feed efficiency compared to those fed a deficient diet (Tesseraud et al., 1999, 2001; Carew et al., 2005; Latshaw, 1993). Due to the importance of dietary lysine, improving the efficiency of lysine use for protein synthesis would prove beneficial to the poultry industry (Manangi et al., 2005). To improve the efficiency of lysine use for protein synthesis, a greater understanding of lysine metabolism would be useful.

Similar to some other amino acids, the catabolic enzymes of lysine metabolism are found in the liver (Higashino et al., 1965). Lysine catabolism by the saccharopine-dependent pathway is thought to be the predominant pathway for lysine degradation in mammals (Broquist H.P., 1991). In this pathway, lysine is converted to saccharopine in an NADPH-dependent step by the enzyme lysine α-ketoglutarate reductase (LKR; E.C. 1.5.1.8). This step involves the condensation of lysine with α-ketoglutarate (α-KG) to form saccharopine (Figure 8). Next, the saccharopine is oxidized by a dehydrogenase to produce α-aminoadipate-γ-semialdehyde and glutamate in an NAD^+-dependent reaction. This reaction is catalyzed by the enzyme saccharopine dehydrogenase (SDH; E.C. 1.5.1.9; Figure 8).

The LKR pathway is thought to be the primary pathway of lysine degradation in rats (Higashino, K., 1965), mice (Papes, F., 1999) chickens (Wang, S., 1973), and fish

(Walton, M.J., 1984). It is also an operational pathway in plants (Arruda, P., 1982). It was first discovered mainly in the liver of rats (Higashino, et. al, 1965), and later specifically in mitochondria (Noda, et al., 1978). When LKR activity in all mitochondrial sub-fractions was examined, LKR was located only in the mitochondrial matrix (Blemings et al., 1994).

The fact that lysine degrading enzymes are located specifically in the mitochondrial matrix indicates that lysine must first be transported into mitochondria, through its inner membrane. The mechanism of lysine transport into the mitochondrial matrix is unclear. Two mitochondrial basic amino acid transporters have been analyzed in *Arabidopsis thaliana*, to better understand how the amino acid arginine, also a basic amino acid, is transported into the mitochondrion for degradation. One of the transporters was purified and determined to have a molecular mass of about 37 kDa (Hoyos et al., 2003). Just as transporting alanine through the plasma membrane is the first, and the rate-limiting step of its metabolism (Fafournoux et al., 1983), it has been hypothesized that the rate of transport of lysine into the mitochondrial matrix also limits its metabolism. A decrease in lysine catabolism has also been correlated with a lysine deficiency (Keiss, 2006).

A deficiency in dietary lysine has proven to be detrimental to the growth of many animal species. Limiting an essential amino acid in a diet will limit protein synthesis, decreasing growth. Chickens (Latshaw, 1993) and fish (Walton et al., 1984) fed lysine-deficient diets had a decrease in weight gain and feed efficiency relative to those fed a lysine-adequate diet. Lysine metabolism is also influenced by dietary lysine. Consumption of a lysine-free diet in rats reduced liver LKR activity (Chu and Hegsted,

1976). Also, it has been hypothesized that the transport of lysine through the inner membrane of the mitochondria limits its catabolism. Therefore, the first objective of the current study was to look for alterations in protein expression, specifically involved in lysine transport, in broilers acclimated to a low lysine diet. The second objective was also to look more globally at alterations in protein expression of the mitochondrial inner membrane with a lysine deficiency.

MATERIALS AND METHODS

Experimental Design

Sixteen day-old Ross x Ross mixed-sex broilers were housed in a group pen. Chickens were fed a standard broiler starter diet ad libitum and provided with nipple drinkers for days 1 through 7 of the study. On day seven, birds were randomly divided into eight groups of two chicks each. Each cage was randomly assigned to be fed either a lysine-adequate diet (1.1% lysine) or lysine-deficient diet (0.73% lysine; Table 7). Each of the groups were fed ad libitum and provided with nipple drinkers. All chickens were housed in metabolism cages. Room temperature was kept around 95^{o}C with a 20:4 light:dark cycle. Pen weights were taken both on day seven and at the termination of the study, days 21 through 24. Feed consumption was determined at the termination of the study. Starting at day 21, two cages were euthanized per day for a period of four consecutive days. Each day, one randomly selected pen receiving a lysine-adequate and one randomly selected pen receiving a lysine-deficient diet were euthanized. Each diet had a total of 4 replications. At the time of euthanasia, livers were removed for further analysis, and stored in isolation medium (70 mM sucrose, 220 mM D-mannitol, 2 mM Hepes and 0.5 mg/mL BSA). All animal protocols were approved by the West Virginia Animal Care and Use Committee (ACUC #05-1203).

Preparation of sub-mitochondrial fractions

Livers from the two birds in each pen were combined for mitochondrial isolation. Mitochondrial sub-fractions were isolated according to the method of Greenawalt (1974). For this procedure, livers were homogenized with a Potter-Elvehjem homogenizer.

Mitochondria were isolated by differential centrifugation from 33% (w/v) whole liver homogenates in isolation medium. The homogenate was centrifuged at 660 rpm for 10 minutes. The resulting supernatant was collected and centrifuged at 9,000 x g for 15 minutes. The pellet, which contained the mitochondria was collected and resuspended in isolation medium. This was centrifuged for a final time at 9,000 x g for 15 minutes and the pelleted mitochondria were then resuspended in isolation medium to give a final concentration of 100 mg of protein per mL. Protein concentrations were determined by the Biuret method. An equal amount of isolated mitochondria and 1.2% digitonin (Sigma Aldrich Inc., St. Louis, MO) in isolation medium were combined, and stirred gently for 15 minutes at 4^{o}C to disrupt the outer membrane. The mitochondria were further diluted with 3 additional volumes of isolation medium. The resultant suspension was centrifuged at 10,000 x g for 10 minutes, leaving the outer membrane and intermembrane space (supernatant) and mitoplasts (pellet). The mitoplasts were further treated with the detergent Lubrol WX (MP Biomedicals LLC, Aurora, OH) at a final concentration of 19 mg/mL to disrupt the inner membrane. This was incubated at 4^{o}C for 15 minutes, and then centrifuged to separate the mitoplasts into matrix and inner membrane at 144,000 x g for 1 hour at 4^{o}C. A protease inhibitor, Halt Protease Inhibitor (Thermo Scientific, Rockford, IL), was added to each sample to prevent protein degradation. Five replicates of the inner membrane fraction were sent to the proteomics lab at the Protein Nutrition Laboratory at Virginia Tech.

2-D Gel Electrophoresis

Protein concentrations were determined for all inner membrane samples using a 2-D Quant kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions. All protein samples were diluted with running buffer (8.125 M urea, 2 M thiourea, 8% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, (50%), (Fisher Scientific, Pittsburgh, PA), 0.2% Bio-Lyte 3/10 Ampholyte (Bio-Rad Laboratories, Hercules, CA), 200 mM tributylphosphate (Bio-Rad), 50 mM dithiothreitol (Bio-Rad)) to 1 µg/µL to ensure equal loading. Protein samples were loaded into the appropriate isoelectric focusing (IEF) tray for immobilized protein gradient (IPG) strips (ReadyStrip, 18cm, pH 3-10 NL; Bio-Rad). A reference standard was also analyzed for a low and high isoelectric point, as well as low and high molecular weight. All sample trays were loaded into a PROTEAN IEF cell (Bio-rad) for electrophoresis. The IPG strips were re-hydrated for 13 hours at 50 V at 20^{o}C, followed by focusing from 0 to 60,000 volts at 20^{o}C for 11 hours. After isoelectric focusing, the IPG strips were equilibrated with 3 mL of equilibration buffer (EB). EB1 contained 6M urea, 20% SDS, 18.5% 2M, pH 8.8 Tris HCl, 20% glycerol. EB 2 contained 130 mM DDT in EB 1, while EB 3 contained 130 mM iodoacetamide in EB 1. After each equilibration, strips were placed on a shaker at room temperature for 30 minutes in buffer. Next, the IPG strips rinsed in 1X TGS buffer (12.5 mM Tris Base, 96 mM glycine, 1.75 mM SDS) were loaded on top of a 2-D gel which had an increasing gradient from 8 to 16% acrylamide. The strips were set into place with 0.5% agarose with Bromophenol blue. All gels were placed in a PROTEAN plus Dodeca Cell tank (Bio-Rad), which contained 1X TGS buffer. The second dimension electrophoresis was performed at 100 V for 15-16 hours

with a BioRad Power Pack 200, until the bromophenol blue was 2cm from the end of the gel. Gels were fixed in 500 mL fixing solution (40% ethanol, 10% glacial acetic acid in water) overnight. All gels were then stained with Flamingo Fluorescent Gel Stain (Bio-Rad) for 3 hours, and washed for 10 minutes. Gels were then scanned and visualized using a Molecular Imager FX Pro Plus MultiImager System (Bio-Rad) and analyzed with PD-Quest 2D gel analysis software (Bio-Rad). All gels were individually packaged in fixing solution in an air tight plastic wrap and stored at room temperature.

Spot Excision

A spreadsheet of protein spots that differed by diet was generated. The adequate to deficient spot density ratios were recorded, and spots with the greatest ratio, as well as a molecular weight similar to the basic amino acid transporter (37kDa) were chosen. Ninety-six spots ranging from 28-48 kDa were selected for excision and mass spectrometric analysis, which was performed at the Virginia Bioinformatics Institute. Spots of interest were excised by a robotic cutter with a 1.5-mm (i.d.) needle tip (Bio-Rad), and placed in a well on a 96-well plate which contained 100 μL of 0.1% acetic acid. Each protein spot was then trypsin digested, and analyzed by mass spectrometry to identify the protein.

Statistics

Significance was assessed using analysis of variance using the PC-SAS General Linear Models procedure. A randomized complete block design was used. When

significant differences occurred ($P<0.05$), means were separated by the least squares mean procedure.

RESULTS

Performance Analysis

The initial weight of chickens was not different (Table 8). There was a 63% decrease in weight gain per day for the chickens consuming the deficient versus the adequate diet (Table 8). Also, while consuming the lysine deficient diet, chickens consumed less feed per day than the adequate-fed counterparts (Table 8). Due to this decrease in feed intake, there was no difference in feed efficiency between diets (Table 8).

Proteomic Analysis

Proteomic analysis was performed on the inner-membrane proteins. Approximately 1,500 protein spots were detected and aligned on all 2-D gels and the density of each spot was analyzed. For analysis, the spot ratios from the four replicates per treatment were averaged. A ratio of average spot densities was calculated for each spot comparing the gels representing the chickens fed the adequate diet compared to those fed the deficient diet. This ratio served as a unit of selection. Spots with the highest ratios were considered, possibly indicating a down-regulation of a potential transport protein while consuming a deficient diet. Spots were also chosen based on molecular weight, to be close to 37 kDa (Appendix 2).

Selected spots were analyzed and assigned a protein name, protein score and protein score confidence interval (Appendix 3). A protein score is a reflection of the possibility that the identified protein is something else. Scores greater than 200 are well defined, 100-200 are acceptable, and below 100 are usually suspect. A protein score

confidence interval is directly related to the protein score. For protein scores less than 70, the confidence interval is zero.

Proteins involved in transport were assessed to determine a possible protein involved in the transport of lysine across the inner membrane of the mitochondria. The proteins involved in transport that were affected by the lysine deficient diet were a voltage-dependent anion-selective channel protein, with a protein score of 114, a voltage gated potassium channel, KCNQ, with a protein score of 66, and finally a ABC transporter, with a protein score of 46.3 (Table 9). Other proteins affected by the lysine deficiency were glutamine synthetase, NADH dehydrogenase, ATP synthase, and Acyl Coenzyme-A dehydrogenase (Table 9), with protein scores of 218, 212, 353 and 148, respectively.

DISCUSSION

The first objective of this study was to investigate alterations in the expression of proteins potentially involved in lysine transport across the inner membrane of mitochondria in broilers acclimated to a low lysine diet. Many proteins involved in transport were affected by the diet. The voltage-dependent anion-selective channel protein is involved in the flux of negatively charged metabolites across the outer membrane of the mitochondrion, such as phosphate, chloride and adenine nucleotides. These proteins have also been associated with the adenine nucleotide translocator of the mitochondrial inner-membrane (Sampson et al., 1997). Voltage-dependent anion channels in their open states have also been shown to control the flow of ATP from the cytosol to mitochondrial spaces, indicating a possible control over mitochondrial function (Rostovtseva and Colombini, 1997). Although this protein is not involved in lysine transport it may be involved in growth via controlling mitochondrial function.

Another transport protein altered by the consumption of a lysine deficient diet was a protein similar to a voltage-gated potassium channel, KCNQ. Voltage-gated potassium channels are a family of ion channels that are activated by intracellular calcium. These proteins are generally associated with action potentials, and are not likely to be involved with lysine transport. ATP-binding cassette transporter, or ABC, proteins were also affected by the lysine deficient diet. This family of proteins is responsible for the translocation of a wide variety of substrates, including sugars, amino acids, metal ions, peptides and proteins (Dean et al., 2001). Many members of the ABC family account for the transport of amino acids and derivatives into and out of living cells (Saier, 2000).

Unfortunately, the protein score and confidence interval for this protein are both low, indicating that we cannot be certain of the protein identity.

The second objective was also to look more globally at alterations in protein expression of the mitochondrial inner membrane with a lysine-deficiency. Glutamine synthetase protein expression in chickens consuming the lysine-deficient diet was only 5% compared to the expression when the chickens were consuming the adequate diet. Glutamine synthetase is the only enzyme responsible for de novo synthesis of glutamine. Glutamine plays an extremely important role in purine biosynthesis in animals, which is accentuated in uricotelic animals, such as chickens (Horn and Featherston, 1972). Glutamine synthetase is usually localized in the cytosol, but it has been discovered in the mitochondrial fractions of the liver in uricotelic species, including the chicken, pigeon and snake (Vorharen and Campbell, 1972). Glutamine synthetase is thought to function by detoxifying ammonia generated intramitochondrially via amino acid catabolism by converting it to glutamine (Vorharen and Campbell, 1972). The increase in glutamine synthetase observed in the inner mitochondrial membrane of broilers consuming a lysine-adequate diet may be due to an increase in amino acids being catabolized.

Complex I and complex V of the electron transport chain were affected by the lysine deficient diet. There was a 30% decrease in Complex I, or NADH dehydrogenase expression in chickens consuming the lysine deficient diet. NADH dehydrogenase is an enzyme that catalyses the transfer of electrons from NADH to coenzyme Q. ATP synthase, or complex V of the electron transport chain had almost a 90% decrease in expression on the inner mitochondrial membrane of chickens consuming the lysine-deficient diet. ATP synthase uses the energy of an electrochemical gradient for the

synthesis of ATP. This 90% reduction of ATP synthase expression may lead to a reduction in the synthesis of ATP, possibly limiting growth. The reduction in the protein abundance of two proteins involved in the electron transport chain may indicate mitochondrial dysfunction.

Since the dominant role of the mitochondria is energy transduction in a cell, mitochondrial dysfunction has been hypothesized to have a great impact on growth performance (Iqbal et al., 2005). The reduction in mitochondrial function associated with decreased feed efficiency and growth may occur because of increased electron leak from the respiratory chain (Bottje et al., 2006). Electron leak leads to the production of reactive oxygen species, which in turn decreases the activity or expression of respiratory chain proteins (Bottje et al., 2004). There is evidence that *in vitro* exposure to reactive oxygen species leads to the impairment of many functional properties of the mitochondria, including an inhibition of complexes in the respiratory chain, especially complexes I and V, and inhibition of adenine nucleotide translocase (Williams et al., 1998). These data may be a sign that the chicken consuming the lysine-deficient diet are under oxidative stress.

Acyl Coenzyme-A dehydrogenase was another protein which had a protein abundance that was four times greater in the chickens consuming the lysine-adequate diet. Acyl CoA dehydrogenase is the enzyme used to catalyze the first step of β-oxidation in the metabolism of fatty acids. This data possibly indicates that chickens consuming the lysine-deficient diet have a decreased ability to metabolize fatty acids compared to those consuming an adequate diet. One explanation may be a potential deficiency of carnitine as a result of the lysine deficiency. Carnitine is synthesized from

products of lysine and methionine metabolism (Tanphaichitr et al., 1976), and it is essential because it plays a role in intramitochondrial transport of fatty acids, as well as initiating β-oxidation of fatty acids (Borum and Broquist, 1977). Thus, a decrease in lysine could lead to a decrease in carnitine and an impaired ability to oxidize fatty acids.

Although we could not confidently determine a protein involved in lysine transport, vast alterations in protein expression on the inner membrane of the mitochondria were due to ingesting a lysine-deficient diet. The impairment of two proteins involved in the electron transport chain, NADH dehydrogenase and ATP synthase may lead to the reduction of the amount of ATP synthesized. If ATP synthesis is decreased, the amount of energy available for growth is also decreased, explaining the reduction in body weight in the lysine deficient birds. It also seems as if broilers consuming a lysine deficient diet had impaired mitochondrial function, possibly leading to oxidative stress.

Figure 8. Initial Enzymatic Reaction of LKR pathway

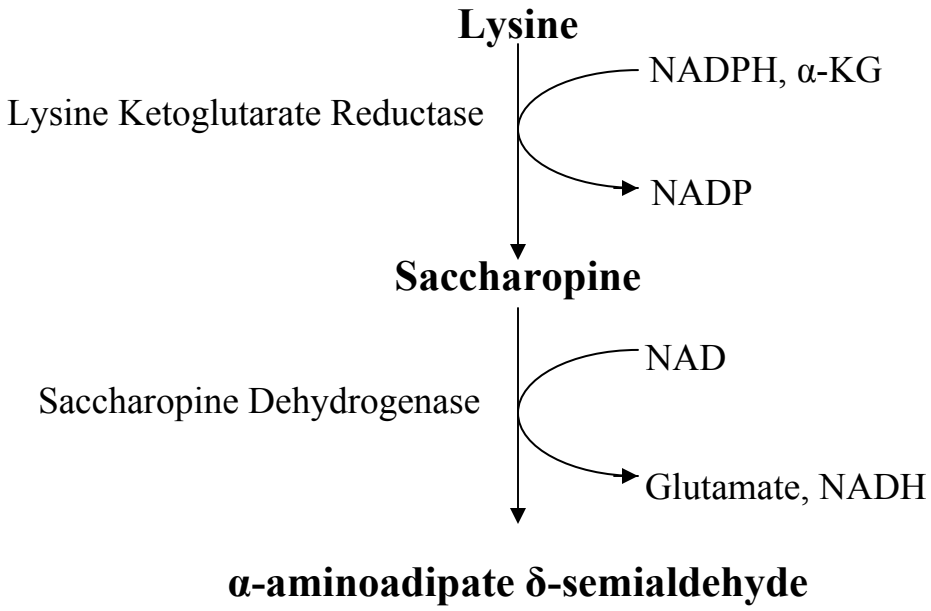

Table 7. Diet Composition on a percentage basis.

Ingredients	Lysine Deficient Diet (%)	Lysine Adequate Diet (%)
Corn Starch	1.37	0.92
Corn	44.49	44.49
Peanut Meal	43.51	43.51
Soybean Oil	5.65	5.65
Limestone, ground	1.4	1.4
Dicalcium Phosphate	2	2
NaCl	0.4	0.4
MB 3000 Premix	0.25	0.25
Choline Chloride	0.1	0.1
L-Valine	0.06	0.06
L-Leucine	0.11	0.11
L-Tryptophan	0.01	0.01
DL-Methionine	0.37	0.37
L-Threonine	0.23	0.23
Coban-60	0.05	0.05
Lysine-HCl	**---**	**0.45**
Total	100	100

Table 8. Performance Analysis

Performance Variable	**LA Diet**	**LD Diet**	**SEM**
Initial Weight (g)	236.38	223.77	7.16
Weight Gain per day (g)	27.65*	16.94	1.97
Feed Consumed per day (g)	49.71*	37.48	2.27
Efficiency	0.561	0.457	0.047

*represents a significant difference with $P<0.05$

Table 9. Protein Identifications

Protein	Ratio	Protein Score	CI	Involvement
Voltage-dependent anion-selective channel protein	1.975	114	100	Membrane Transport
Voltage gated potassium channel	2.300	66	87	Membrane Transport
ABC Transporter	4.268	46.3	N/A	Membrane Transport
Glutamine Synthetase	23.646	218	100	Glutamine Synthesis
NADH Dehydrogenase	1.44	212	100	Electron Transport Chain
ATP Synthase	9.091	353	100	Electron Transport Chain
Acyl CoA dehydrogenase	4.093	148	100	Fatty Acid Oxidation

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Appendix 1. Correlation Matrix

	Wt I	Wt F	ADFI	ADG	Eff	CF	HSI	AE	N Ret	Lipid	mRNA	LKR A	LOX	OC
Wt F	**0.806**[1]													
P Value	**<0.0001**													
ADFI	**0.409**	**0.708**												
P Value	**0.001**	**<0.0001**												
ADG	**0.541**	**0.921**	**0.748**											
P Value	**<0.0001**	**<0.0001**	**<0.0001**											
Eff	-0.074	**0.391**	0.213	**0.612**										
P Value	0.579	**0.002**	0.109	**<0.0001**										
CF	**0.349**	**0.618**	**0.376**	**0.645**	**0.529**									
P Value	**0.007**	**<0.0001**	**0.004**	**<0.0001**	**<0.0001**									
HSI	0.067	-0.113	0.016	-0.183	**-0.421**	**-0.407**								
P Value	0.616	0.398	0.904	0.167	**0.001**	**0.002**								
AE	-0.034	-0.137	0.173	-194	-0.237	-0.118	**0.43**							
P Value	0.801	0.304	0.898	0.145	0.073	0.377	**0.0008**							
N Ret	**-0.268**	0.197	0.138	**0.444**	**0.742**	**0.522**	**-0.479**	-0.197						
P Value	**0.042**	0.139	0.300	**0.0005**	**<0.0001**	**<0.0001**	**0.0001**	0.138						
Lipid	**0.413**	**0.287**	**0.308**	0.138	**-0.324**	-0.128	**0.309**	0.031	**-0.33**					
P Value	**0.001**	**0.029**	**0.018**	0.302	**0.013**	0.338	**0.018**	0.816	**0.011**					
mRNA	0.071	0.191	0.226	0.211	0.097	0.007	-0.032	-0.016	0.176	0.063				
P Value	0.611	0.169	0.103	0.130	0.490	0.962	0.819	0.909	0.207	0.654				
LKR A	-0.18	-0.124	0.031	-0.079	-0.008	-0.124	0.011	0.012	-0.039	-0.206	-0.136			
P Value	0.176	0.354	0.817	0.555	0.955	0.355	0.934	0.93	0.772	0.120	0.332			
LOX	-0.095	-0.152	-0.152	-0.124	-0.097	-0.149	-0.021	-0.154	-0.107	0.073	0.08	0.077		

P Value	0.518	0.297	0.298	0.396	0.508	0.306	0.887	0.290	0.464	0.616	0.601	0.598		
OC	**-0.399**	**-0.336**	-0.255	-0.243	-0.059	-0.232	0.049	-0.257	-0.086	-0.071	-0.005	0.057	**0.375**	
P Value	**0.002**	**0.011**	0.058	0.072	0.668	0.086	0.721	0.056	0.530	0.601	0.972	0.677	**0.0094**	
NEFA	0.195	0.029	-0.064	-0.101	**-0.271**	-0.229	-0.088	**-0.3**	**-0.288**	0.096	0.123	0.012	0.005	0.024
P Value	0.150	0.832	0.635	0.457	**0.0437**	0.090	0.519	**0.025**	**0.031**	0.481	0.388	0.932	0.975	0.863

[1]This column represents the R-value of the correlation analysis of pooled data

Wt I- Initial Weight
Wt F- Final Weight
ADFI- Average Daily Feed Intake
ADG- Average Daily Gain
Eff- Feed Efficiency
CF- Condition Factor
HSI- Hepatosomatic Index
AE- Ammonia Excretion
N Ret- Nitrogen Retention
Lipid- Lipid Content
mRNA- LKR mRNA Abundance
LKR A- LKR Activity
LOX- Lysine Oxidation
OC- Oxygen Consumption
NEFA- Non-esterified Fatty Acids

Appendix 2. Spot Densities

Number[1]	Molecular Mass (kDa)	Isoelectric Point[2]	A:D ratio of spot density[3]
1	41.2	6.58	23.646
2	32.93	3.79	9.091
3	31.82	5.55	7.412
4	31.91	4.17	6.841
5	31.61	5.33	6.603
6	42.91	4.24	5.608
7	39.44	-1	4.801
8	37.25	9.09	4.309
9	39.17	6.84	4.268
10	38.98	5.75	4.191
11	32.05	6.85	3.918
12	42.07	5.34	3.885
13	42.73	5.6	3.336
14	39.07	-1	3.230
15	37.42	5.64	3.032
16	33.96	4.52	3.033
17	43.1	5.19	4.806
18	45.98	5.11	4.449
19	44.09	5.31	4.159
20	44.2	5.5	4.093
21	33.44	5.72	3.273
22	37.89	6.85	2.945
23	34.71	4.8	2.866
24	30.11	8.33	2.859
25	37.49	5.8	2.769
26	38.88	6.59	2.711
27	34.58	8.92	2.585
28	37.87	6.07	2.586
29	39.44	5.81	2.607
30	38.42	5.46	2.609
31	33.09	3.47	2.361
32	39.92	6.98	2.334
33	47.22	5.19	2.312
34	34.19	8.78	2.300
35	37.33	5.83	2.287
36	33.67	5.76	2.266
37	37.76	6.61	2.194
38	37.23	7.1	2.210
39	39.13	6.98	2.170
40	34.96	5.46	2.061

41	38.36	8.94	2.010
42	35.07	5.64	1.968
43	45.33	5.66	1.975
44	40.87	6.6	1.954
45	39.23	6.57	1.871
46	33.49	8.52	1.838
47	34.27	5.33	1.841
48	47.2	6.58	13.915
49	29.04	8.98	10.149
50	29	8.91	9.274
51	46.82	5.74	3.674
52	47.55	4.58	3.621
53	30.2	6.56	3.280
54	46.28	6.95	2.872
55	33.4	3.69	2.792
56	40.76	4	2.087
57	35.48	6.77	1.821
58	41.91	5.19	1.763
59	34.45	6.88	1.736
60	33.7	6.69	1.722
61	37.85	6.72	1.716
62	30.95	5.41	1.699
63	41.08	4.43	1.693
64	39.1	5.99	1.698
65	32.18	6.95	1.667
66	37.73	5.72	1.660
67	30.53	5.59	1.643
68	37.79	6.94	1.652
69	38.24	6.55	1.628
70	28.09	5.18	1.629
71	38.23	5.89	1.630
72	37.98	6.57	1.631
73	30.41	6.86	1.615
74	29.52	5.16	1.608
75	39.36	5.6	1.582
76	30.64	5.75	1.574
77	35.62	6.92	1.565
78	32.21	5.91	1.567
79	31.52	6.72	1.567
80	39.88	6.8	1.533
81	37.28	5.27	1.519
82	39.54	8.63	1.504
83	38.36	7.41	1.508

84	38.06	-1	1.488
85	39.79	6.64	1.479
86	37.92	7.21	1.459
87	35.22	-1	1.454
88	20.8	5.13	4.183
89	24.96	6.68	4.097
90	25.63	6.57	1.715
91	47.67	7.46	1.450
92	29.25	4.92	1.445
93	39.9	6.89	1.438
94	40.81	4.61	1.439
95	46.14	5.2	1.396
96	38.49	5.38	1.386

[1] Number corresponding to the order of spots chosen for analysis
[2] An isoelectric point of -1 means it is less than 3 or greater than 10
[3] A: D Spot densities are the spot density ratios of the pooled average for the gels for the chickens fed the adequate diet by the pooled average for the gels for the chickens fed the lysine deficient diet.

Appendix 3. Spot Identification

Spot[1]	Score[2]	CI %[3]	Protein Identifications[4]
1	**318**	**100**	**Glutamine synthetase**
2	**353**	**100**	**ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1**
3	51	0	Neuroregulin-1 (increases phosphorylation on tyrosine residues)
	36.7	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	35.3	0	membrane-type matrix metalloproteinase 3 [Homo sapiens]
	33.1	0	serine protease
4	54.8	0	MAP kinase
	38.6	0	keratin
5	**443**	**100**	**prohibitin- charperones respiratory proteins into matrix**
6	34	0	wingless-type MMTV integration site family 3
	25.7	0	KCNQ potassium channel (Voltage-gated)
	27.4	0	putative ABC transporter (ATP-binding protein) ATP-binding cassette transporter
	26.8	0	olfactory binding protein
	26.7	0	membrane tyrosine phosphatase
	24.8	0	mitochondrial ribosomal protein L42 isoform a (protein synthesis)
7	51.7	0	O. volvulus cyclophilin 4
	48.5	0	X Kell blood group
	40.3	0	C-type lectin domain family 4, member g [Mus musculus]
	39.6	0	elongation factor 1-alpha [Ctenolepisma lineata]
	38.8	0	glutamine synthetase [Mus musculus]
	38.7	0	myelin protein zero [Equus caballus]
8	50.7	0	MAP kinase
	45.9	0	Neuroregulin-1
	43.5	0	C-type lectin domain family 4, member g [Mus musculus]
	37.1	0	methyltransferase-like protein
9	52.7	0	Neuroregulin-1
	46.9	0	ABC transporter
	46.3	0	glycoprotein A-rich protein
	41.4	0	defensin
	40.3	0	similar to phosphoenolpyruvate-protein phosphotransferase
	38.9	0	DNA-damage-inducible transcript 3 [Homo sapiens]
10	**413**	**100**	**keratin**
11	77.7	0	X Kell blood group
	38.5	0	Paraneoplastic antigen Ma3

	37.4	0	MAP kinase
	36.6	0	sodium bicarbonate cotransporter 4f [Homo sapiens]
	36.5	0	similar to phosphoenolpyruvate-protein phosphotransferase
12	35.5	0	methyltransferase like protein, transferes methyl groups
	29.3	0	cyclin-dependent kinase inhibitor 1C
	27.4	0	acyl-Coenzyme A dehydrogenase, (1st step in beta-oxidation)
	26.2	0	succinate-Coenzyme A ligase, ADP-forming, beta subunit [Mus musculus]
13	28.9	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
	34.3	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	30.8	0	5-HT7-like serotonin receptor [Taeniopygia guttata]
	30.7	0	Glycosyltransferases
14	47.2	0	Neuroregulin-1
	40.1	0	trypsin
15	57.8	0	phosphoenolpyruvate carboxykinase [Gallus gallus]
	48.7	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	37	0	methyltransferase-like protein
	35.4	0	multidrug resistance protein 1 [Canis familiaris]
	34.7	0	elongation factor 1-alpha [Papilio thoas]
16	59.1	0	elongation factor-1 alpha [Pedetontus saltator]
	50.6	0	trypsin
17	61.7	60	encoded by nuclear genes and help in protein synthesis within the mitochondrion.
	61.3	0	acyl-Coenzyme A dehydrogenase,
	36.4	0	elongation factor 1-alpha
18	43.2	0	similar to ABC transport permease
	38.7	0	elongation factor 1-alpha
19	10.6	0	Ig gamma-1 CH3 domain [Mus musculus]
	9.9	0	mitochondrial ribosomal protein S14 [Homo sapiens]
	9.9	0	defensin
	9.9	0	homeodomain
20	**148**	**100**	**acyl-Coenzyme A dehydrogenase**
	43.8	0	solute carrier organic anion transporter family, member 6d1
21	50.8	0	MITF protein [Mus musculus]
	46.7	0	elongation factor-1 alpha [Pedetontus saltator]
	36.7	0	cAMP-responsive element binding protein 2 [Lymnaea stagnalis]
	33	0	Purkinje cell espin isoform 1 [Rattus norvegicus]
22	41.5	0	mitochondrial ubiquitin ligase of nFkb

	36.6	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	36	0	ABC transporter permease
	33.5	0	connexin 31 [Bos taurus]
	31.9	0	elongation factor-1alpha [Pseudopolydesmus serratus]
	30.2	0	serine protease
23	52.1	0	vertebrate solute carrier family 25 (mitochondrial carrier\; phosphate car
	48.2	0	ATP synthase subunit beta, mitochondrial precursor
24	56.6	0	similar to abhydrolase domain containing 11 isoform 1 isoform 1 [Canis familiaris]
	36.9	0	Ig epsilon-chain - chimpanzee (fragment)
	32.5	0	aminoacylase 1 [Xenopus laevis]
	31.3	0	trimethyllysine hydroxylase, epsilon [Rattus norvegicus]
25	42.1	0	similar to transcription factor 2A
	39.5	0	Neuroregulin-1
	36.7	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	36.7	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
26	**190**	**100**	**3-hydroxyisobutyryl-Coenzyme A hydrolase [Gallus gallus]**
	66.1	86.79	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain [Homo sapiens]
27	**391**	**100**	**PREDICTED: similar to 2,4-dienoyl-CoA reductase [Gallus gallus]**
	66.5	0	Myoinhibiting peptide precursor CG6456-PA [Drosophila melanogaster]
	51.4	0	similar to carnitine deficiency-associated gene
	48.3	0	similar to WNK lysine deficient protein kinase 1
	47.4	0	RNA polymerase II largest subunit [Isohypsibius elegans]
	45.3	0	clathrin, light polypeptide A [Gallus gallus]
28	51.3	0	potassium channel KCNQ [Drosophila melanogaster]
	48.6	0	cation transmembrane transport
29	19.7	0	T-cell receptor gamma subunit [Equus caballus]
	17.9	0	hexokinase [Sparus aurata]
	17	0	neuropeptide Y [Rattus norvegicus]
	16.4	0	plectin isoform plec 1i [Mus musculus]
	16	0	cytochrome oxidase II [Anacharis zealandica]
30	**201**	**100**	**keratin**
31	**113**	**100**	**ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1**
32	9.8	0	vimentin [Mus musculus]
	9.6	0	cytochrome b [Chelonia mydas]

	9.6	0	beta-2-microglobulin [Equus caballus]
	9.6	0	male specific double sex protein
	9.6	0	T cell receptor beta chain variable region [Gallus gallus]
	9.1	0	urotensin I
33	44	0	RNA polymerase II largest subunit [Tanystylum orbiculare]
	41.8	0	sperm adhesion molecule 1 [Rattus norvegicus]
	40.3	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	38.8	0	TOLLIP protein [Homo sapiens]
	38.2	0	whn transcription factor [Branchiostoma lanceolatum]
	37.8	0	hyaluronidase 1 [Mus musculus]
34	**442**	**100**	**PREDICTED: similar to 2,4-dienoyl-CoA reductase [Gallus gallus]**
	114	**100**	**Voltage-dependent anion-selective channel protein 2**
	61.6	0	F-box protein Fbx20 [Homo sapiens]
	56.1	0	GAP-2-5 [Caenorhabditis elegans]
	55.7	0	doublesex isoform M [Bombyx mori]
35	**131**	**99.78**	**ribosomal protein P0 [Gallus gallus]**
36	**80.2**	**99.5**	**KIAA0103 protein [Mus musculus] (tetratricopeptide repeat domain 35)**
	45.9	0	Wnt3 protein [Ciona intestinalis]
	37.4	0	serine-threonine kinase 9, phosphorylate the OH group of serine or threonine
37	54.3	0	trypsin
38	**349**	**100**	**crystallin, zeta (quinone reductase) [Gallus gallus]**
	57.5	0	apoptosis-inducing, TAF9-like domain 1 isoform C [Homo sapiens]
	51.5	0	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)
	44.4	0	tumor-suppressor [Equus caballus]
	43.8	0	whn transcription factor [Branchiostoma lanceolatum]
39	54.4	0	nueroregulin -1
	31.5	0	SMDF neuregulin alpha 2b [Rattus norvegicus]
	31.3	0	eukaryotic translation intiation factor 4GI [Felis catus]
40	45.5	0	PREDICTED: similar to Glycine N-methyltransferase isoform 1 [Canis familiaris]
	29.6	0	cytochrome b [Dendropoma nebulosum]
	29.4	0	prostaglandin D synthase [Oryctolagus cuniculus]
	25.1	0	p53 [Mus sp.]
41	43.9	0	similar to Membrane bound O-acyl transferase, MBOAT
	43.1	0	phosphotyrosyl phosphatase activator [Homo sapiens]

	38.9	0	calnexin [Homo sapiens]
	34.2	0	U11/U12 snRNP 35K isoform a [Homo sapiens]
	31.9	0	Activator of G protein Signalling family member (ags-3) [Caenorhabditis elegans]
	30.2	0	centromeric histone [Drosophila lucipennis]
42	**97.1**	**99.9**	**keratin 1 [Homo sapiens]**
	44.7	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	44.4	0	whn transcription factor [Branchiostoma lanceolatum]
	41.6	0	ACyLtransferase-like family member (acl-13) [Caenorhabditis elegans]
43	66.3	87	potassium channel KCNQ , voltage gated potassium channel
	63.7	77	KCNQ potassium channel CG33135-PB, isoform B [Drosophila melanogaster]
	52.6	0	Neuroregulin-1
	47.4	0	whn transcription factor [Branchiostoma lanceolatum]
	45.1	0	elongation factor-1 alpha [Machiloides banksi]
44	**150**	**100**	**glutamine synthetase [Gallus gallus]**
	309	**100**	**acyl-Coenzyme A dehydrogenase, long-chain [Mus musculus]**
45	**140**	**100**	**acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain [Sus scrofa]**
46	**104**	**99.99**	**ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1**
47	40.9	0	Wnt3 protein [Ciona intestinalis]
	39.7	0	COBRA1 protein [Homo sapiens]
	38.9	0	C-type lectin domain family 4, member g [Mus musculus]
	35.8	0	whn transcription factor [Branchiostoma lanceolatum]
	35.4	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	33.2	0	toll interacting protein [Homo sapiens]
48	71.5	96	acyl-Coenzyme A dehydrogenase, long chain [Gallus gallus]
49	66.1	0	glioblastoma amplified sequence [Gallus gallus]
	64.1	0	similar to NipSnap2 protein (associated with brain tumors)
	49.2	0	ventral anterior homeobox containing gene 1 [Rattus norvegicus]
50	**259**	**100**	**keratin**
51	50.4	0	NADH dehydrogenase (ubiquinone) Fe-S protein 2 [Rattus norvegicus]
	26.4	0	high affinity IgE receptor beta subunit [Homo sapiens]
	20.9	0	Ubiquitin carboxyl-terminal hydrolase 35 (Ubiquitin thioesterase 35)

52	**114**	**100**	**ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide**
53	46.1	0	hydrogen voltage-gated channel 1 [Gallus gallus]
	43.4	0	Methyltransferase like 2A [Homo sapiens]
	43.2	0	MITF protein [Mus musculus]
	42	0	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10 [Rattus norvegicus]
	38	0	olfactory binding protein [Leucophaea maderae]
54	**298**	**100**	**pyruvate dehydrogenase kinase, isozyme 3 [Gallus gallus]**
	60.8	0	myofibrillogenesis regulator 1 isoform 1
55	**213**	**100**	**p32 subunit of splicing factor SF2 [Gallus gallus]**
	93.8	**99.98**	**glycine C-acetyltransferase, participates in glycine, serine and threonine metabolism**
	58.8	0	Microfibrillar associated protein 5 [Mus musculus]
	50.1	0	spermidine/spermine N1-acetyl transferase 1 [Danio rerio]
56	30.2	0	voltage gated potassium channel
	27.3	0	acyl-coA dh, catalyzes the 1st step in beta-oxidation
	25.3	0	Huwentoxin-2a precursor (Huwentoxin-IIa)
	22.1	0	Ig kappa chain precursor VJ5C-region
57	**111**	**100**	**Thiosulfate sulfurtransferase (Rhodanese)**
	45.3	0	Homeobox protein Hox-A7
	43.6	0	chorion factor CF1 [Drosophila melanogaster]
58	11.4	0	NADH dehydrogenase subunit 4 [Conolophus subcristatus]
	9.6	0	transcription factor AP-2 gamma [Homo sapiens]
	9.6	0	vimentin [Mus musculus]
	9.6	0	male specific double sex protein
	9.6	0	beta-2-microglobulin [Equus caballus]
59	55.1	0	Neuroregulin-1
	42.4	0	insulin-like growth factor II [Mugil cephalus]
	42.4	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
	40.4	0	MITF protein [Mus musculus]
	39.2	0	elongation factor-1 alpha [Enchenopa binotata]
60	15	0	ATP synthase F0 subunit 8 [Camponotus laevigatus]
	14.9	0	mind bomb [Danio rerio]
	14.7	0	putative Ash protein [Archispirostreptus sp. HD-2003]
	14.5	0	ubiquitin ligase mind bomb [Mus musculus]
61	45.6	0	trypsin
	42	0	putative phosphatidyl-inositol-4-phosphate 5-kinase [Drosophila melanogaster]

	41	0	Serpentine Receptor, class H family member (srh-141) [Caenorhabditis elegans]
	37.7	0	Neuroregulin-1
	36.4	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
62	10.1	0	T-cell receptor beta chain V-J region [Mus sp.]
	9.8	0	Potassium channel toxin alpha-KTx 5.2 (Leiurotoxin I-like toxin P05) (AmP05)
	9.8	0	doublesex-related protein Dmrt15 [Coturnix coturnix]
	9.7	0	glue protein
	9.6	0	huwentoxin-I [Selenocosmia huwena]
	9.5	0	metalloprotease/disintegrin xMDC11.1 [Xenopus laevis]
63	**200**	**100**	**laminin receptor**
	200	**100**	**similar to 40S ribosomal protein SA (P40) [Bos taurus]**
64	65.6	81	3-hydroxyisobutyryl-Coenzyme A hydrolase [Gallus gallus]
	49.5	0	Neuroregulin-1
	47.5	0	Myoinhibiting peptide precursor CG6456-PA [Drosophila melanogaster]
	45.5	0	glutamate-ammonia ligase (EC 6.3.1.2) - Chinese hamster
65	35.9	0	serine protease
66	26.3	0	testis expressed 9 [Homo sapiens]
	23.4	0	Chriz [Drosophila mauritiana]
	22.3	0	general transcription factor IIA, 2, 12kDa [Homo sapiens]
	22.2	0	dipeptidase 2 [Mus musculus]
67	54	0	elongation factor-1 alpha [Pedetontus saltator]
	46.3	0	ribosomal protein SA [Homo sapiens]
	46.3	0	laminin-binding protein
68	48.4	0	potassium channel, subfamily K, member 10 isoform 2 [Homo sapiens]
	45.9	0	calctonin receptor like protein [Rana catesbeiana]
	39.2	0	interferon gamma
	39.1	0	espin isoform 4 [Mus musculus]
	38.7	0	eIF2B-alpha CG7883-PA [Drosophila melanogaster]
69	50	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	40.9	0	Neuroregulin-1
	36	0	Serine/threonine kinase of the haspin family, protein serine/threonine phosphate
	37.7	0	Myosin-XV (Unconventional myosin-15)
70	44	0	serine protease
71	**106**	**99.99**	**similar to Coproporphyrinogen oxidase**

	45	0	Luciferin 4-monooxygenase (Luciferase)
	42.8	0	C-type lectin domain family 4, member g [Mus musculus]
	42.7	0	eppin [Oryctolagus cuniculus]
	42.6	0	elongation factor-1 alpha [Ophiderma grisea]
	39	0	Neuroregulin-1
72	13.6	0	immunoglobulin H-chain V-region [Mus musculus]
	11.5	0	CLIP-associating protein CLASP2 [Homo sapiens]
	11.1	0	beta-2-defensin
	10.9	0	cytochrome c oxidase subunit I [Sphenodon punctatus]
	10.8	0	cyclic AMP-dependent protein kinase catalytic subunit [Caenorhabditis elegans]
73	**78.5**	**99.23**	**Endoplasmic reticulum protein ERp29**
	73.5	**97.6**	**es1 protein [Gallus gallus]**
	50.3	0	THAP domain containing 4 [Mus musculus]
	47.1	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	42.4	0	Neuroregulin-1
	41	0	sodium bicarbonate cotransporter 4f [Homo sapiens]
	39.8	0	elongation factor-1 alpha [Taygetis virgilia]
74	**140**	**100**	**similar to metaxin 2 isoform 2**
	49.6	0	membrane-type matrix metalloproteinase 3 [Homo sapiens]
	46.4	0	phosphotyrosyl phosphatase activator [Oryctolagus cuniculus]
	44.1	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
75	66.7	88.5	TPA: TPA_exp: neuregulin 1 isoform ndf43 [Homo sapiens]
	54.3	0	acyl-Coenzyme A dehydrogenase, short/branched chain [Gallus gallus]
	43	0	whn transcription factor [Branchiostoma lanceolatum]
	39.1	0	adducin 1 (alpha) isoform 1 [Mus musculus]
76	**105**	**99.99**	**coenzyme Q5 homolog, methyltransferase [Gallus gallus]**
	60.5	52	acyl-Coenzyme A dehydrogenase, long chain [Danio rerio]
77	**219**	**100**	**Thiosulfate sulfurtransferase (Rhodanese)**
	72.5	96.97	G protein-regulated inducer of neurite outgrowth 1 [Mus musculus]
	67.7	90	GRIN1 [Mus musculus]
	54.8	0	X Kell blood group precursor-related family, member 3 [Homo sapiens]
	54.2	0	clathrin light chain [Drosophila melanogaster]
	52.7	0	elongation factor-1 alpha [Enchenopa binotata]
	51.5	0	6-phosphogluconate dehydrogenase [Sphaerium simile]

	61.2	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	49.2	0	Neuroregulin-1
	45.6	0	C-type lectin domain family 4, member g [Mus musculus]
	42.7	0	U11/U12 snRNP 35K isoform a [Homo sapiens]
79	9.9	0	Antimicrobial peptide THP3 (Turkey heterophil peptide 3)
	9.9	0	T-cell receptor beta chain [Homo sapiens]
	9.8	0	Hox 3 homeodomain protein [Priapulus caudatus]
	9.7	0	filensin [Mus musculus]
	9.7	0	20 kda phosphorylation-dependent protein phosphatase-1 inhibitory protein
	9.6	0	beta-fibrinogen [Icterus bullockii]
80	45.2	0	Neuroregulin-1
	44	0	F-box protein Fbx20 [Homo sapiens]
	42.8	0	putative elongation factor 1-alpha [Crenobia alpina]
81	52.9	0	Neuroregulin-1
	48.1	0	angiopoietin-like 3 [Danio rerio]
	46.2	0	protein phosphatase 2A, regulatory subunit B' isoform b [Homo sapiens]
	44.7	0	phosphotyrosyl phosphatase activator [Oryctolagus cuniculus]
82	**319**	**100**	**aldolase B, fructose-bisphosphate [Gallus gallus]**
	121	**100**	**Aspartate Aminotransferase**
	104	**99.99**	**fructose 1,6 biphosphate aldolase B [Chaetodon citrinellus]**
83	**151**	**100**	**glyceraldehyde-3-phosphate dehydrogenase [Gallus gallus]**
	62.3	0	MITF protein [Mus musculus]
	57.9	0	GluClalpha2B protein [Caenorhabditis elegans]
84	**91.9**	**99.9**	**glyceraldehyde-3-phosphate dehydrogenase [Gallus gallus]**
	49.2	0	MAP Kinase family member (mpk-2) [Caenorhabditis elegans]
	45.7	0	membrane-type matrix metalloproteinase 3 [Homo sapiens]
	44.7	0	potassium channel, KvQLT family member (kqt-3) [Caenorhabditis elegans]
	44.2	0	MITF protein [Mus musculus]
85	**129**	**100**	**mitochondrial ribosomal protein L39**
	63.9	0	potassium channel KCNQ
	48.4	0	vulcan CG8390-PD, isoform D [Drosophila melanogaster]
	47.4	0	potassium inwardly-rectifying channel, subfamily J, member 8 [Danio rerio]
86	64	0	PREDICTED: similar to aldose reductase [Gallus gallus]
	39.9	0	t-cell receptor alpha
	37.2	0	Zinc finger, FYVE domain containing 20 [Mus musculus]

	37	0	coronin, actin binding protein, 1A [Homo sapiens]
	36.2	0	olfactory binding protein [Leucophaea maderae]
87	**409**	**100**	**prohibitin 2 [Gallus gallus]**
88	11.7	0	male accessory gland protein [Drosophila melanogaster]
	10.8	0	NADH dehydrogenase subunit 4 [Conolophus subcristatus]
	10.7	0	gastric mucin [Mus musculus]
	10	0	leucokinin precursor [Drosophila melanogaster]
	10	0	myosin II, (first expressed exon)
	10	0	filensin [Mus musculus]
89	41	0	whn transcription factor [Branchiostoma lanceolatum]
	36.3	0	zinc finger protein 616 [Homo sapiens]
	34.1	0	triosephosphate isomerase 2 [Philodina roseola]
	33.5	0	haspin [Mus musculus]
	32.6	0	haploid germ cell-specific nuclear protein kinase [Mus musculus]
90	63.8	77	Ecdysone-induced protein 75 B
	61.1	0	catalytic subunit of DNA polymerase zeta
	57.7	0	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog [Homo sapiens]
	55.9	0	Methyltransferase like 2A [Homo sapiens]
	54.5	0	ventral anterior homeobox containing gene 1 [Rattus norvegicus]
	48.1	0	integral membrane protein 2B, like [Danio rerio]
91	**127**	**100**	**PREDICTED: similar to Thiolase-prov protein [Gallus gallus]**
	61.1	0	matrix metalloproteinase 16 isoform 1 preproprotein [Homo sapiens]
	59.1	0	mitochondrial trifunctional protein, beta subunit [Bos taurus]
92	**213**	**100**	**NADH dehydrogenase (ubiquinone)**
93	**96.3**	**99.98**	**glycine C-acetyltransferase**
	43.6	0	FSHR (mammalian follicle stimulating hormone receptor) homolog family member (fshr-1)
	41.4	0	eukaryotic translation initiation factor 4, gamma 1 isoform b [Mus musculus]
	38	0	elongation factor-1 alpha [Malacosoma californicum]
	34.6	0	angiotensin-converting enzyme [Rattus norvegicus]
94	34.4	0	Myelin P0 protein precursor (Myelin protein zero) (Myelin peripheral protein) (MPP)
	32.5	0	Neuroregulin-1
	30.7	0	phosphotyrosyl phosphatase activator [Homo sapiens]
	29.5	0	GLoBin family member (glb-6) [Caenorhabditis elegans]
95	10	0	Neurotensin (NT)

	9.9	0	filensin [Mus musculus]
	9.8	0	t-cell receptor
	9.8	0	IP=20 kda phosphorylation-dependent protein phosphatase-1 inhibitory protein
	9.8	0	bombesin receptor subtype 3 [Homo sapiens]
	9.6	0	DNA-binding protein HOX9 [Holopneustes purpurescens]
	9.5	0	homeodomain protein [Girardia tigrina]
96	66	99.35	isocitrate dehydrogenase 3 (NAD+) alpha [Rattus norvegicus]

[1] Number corresponding to the order of spots chosen for analysis
[2] Protein score- It is a reflection of the possibility that the identified protein is something else. Scores greater than 200 are well defined, 100-200 are acceptable, and below 100 are usually suspect.
[3] Confidence Interval- A confidence interval score that is directly related to the protein score. For protein scores less than 70, the CI is zero.
[4] Identified proteins

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